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VACCINE DEVELOPMENT AGAINST PLAGUE, GLANDERS AND MELIOIDOSIS IN THE FORMER SOVIET UNION IN COMPARSION TO THE CURRENT STATE OF GLOBAL KNOWLEDGE

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DEDICATION

This work is dedicated to my parents and my daughter.

Losing a loved one is one of the most difficult things we can go through, so much more if it is a parent. No words can express what I am feeling right now and I'm sure only time can help me accept the fact. My father Kissi Jimaa Biraatu was one of those hard working fathers that always got his way. His words were the law in our house as well as in our community. He thought me one of the most valuable lessons for life, standing up for what you believe in. Don't let anybody tell you otherwise. As I grew older, I slowly understood what he means. And I also discovered that he was a very compassionate and kind person. Today I could have been the happiest person, if he stands besides me and can see his sons endeavour. His saying: "*Obsaan*[1] *Qaalomu*[2] *"* an Oromo proverb, which literally means "Those who have the ability to withstand hardship can be *qaalu*", has always been the principal guide line of my life and accomplishment and will be in future. Let his soul rest in peace in "*Mana Waaqa*[3]".

My Mother Aberu Tolcha Bule, the strongest mother whose life is devoted for her children, the loveliest of all not only in our own family but also in our community, for her unconditional support and encouragement we all here children granted to determine our future. She is a peace maker and peace lover in our village. She always tell as *Waaqa* loves truth and reconciliation. She is a pillar of our family, thought us the cradle of our unique socio political system "*Aadaa Gadaa*", of the Oromo people. The bases of my stability, pyschological make-upand contemplation. She thought us our culture, tradition, religions rites, norms, taboos, adages, songs dances, celebrations, puzzles, games, conflict management e.t.c, what she inherited. That is who I am today.

My beautiful daughter, Tsion, whose love, passion always inspires my life, invigorates my spirit of hopes and energy for my accomplishments.

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[1] *Obsaa* literarily means Patience
[2] *Qaalu* is the highest Spiritual Stage one can be or achieve in Gaada system
[3] *Mana* Waaqa is the home of our creature of Oromo people
[4] *Aa daa Gadaa* is the oldest, unique African democratic and egalitarian socio-political system of Oromo people

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ABBREVIATIONS AND ACRONYMS

AD	Anno Domini
AMR	Aminoglycoside Macrolide Resistance
APC	Antigen Presenting Cell
API	Anti-Plague-Institute
APS	Anti-Plague Stations
ARDS	Adult Respiratory Syndrome
ATCC	American Type Culture Collection
BC	Before Christ
BCG	Bacillus Calmette-Guerin
BW	Biological Weapon
BWP	Biological Weapon Program
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
CFSPH	The center for Food Security an Public Health
CIDRAP	Center for Infectious Disease Research and Policy
CIS	Commonwealth of Independent States
CNS	Center for Nonproliferation Studies
CSDT	Center of Special Laboratory Diagnositc and Treatment
CTL	Cytolytic T Lymphocytes
Dcl (LD100)	Absolute lethal dose (*Dosis certa letalis*)
DIC	Dissiminated Intravascular Coagulopathy
Dlm	Minimal lethal dose (*Dosis letalis minima*)
DNA	Deoxyribonucleic Acid
DSMZ	German Collection of Microorganisms and Cell Cultures
DTH	Delayed Type Hypersensitivity
EIDIOR	Emerging Infectious Disease of the Indian Ocean Rim
ELISA	Enzyme-linked immunoassay
EPS	Exopolysaccharide
e.t.c.	etcetera
EV	Stands for the initials of an European child died of plague
FAO	Food and Agriculture Organization
FIAC	Federal Interagency Centres
FMD	Foot and Mouth Disease
FS	Former Soviet Union
HMWPS	High Molecular Weight Proteins
ICNB	International Code of Nomenclature of Bacteria
IFNα	Interferon-alpha
IFNγ	Interferon-gama
IgG	Immunoglobulin G

IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
ImD50	Dose of vaccine strain protecting 50 % of infected animals
i.m.	Intramuscular
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
kDa	Kilo-dalton
KWC	Killed-whole-cell
LD_{50}	Minimal lethal dose that kills 50 % of infected experimental animals
LPS	Lipopolysaccharide
LTbV	Live tuberculosis vaccine
LTV	Live tularemia vaccine
mAb	Monoclonal antibody
mDA	Mega-dalton
MeSH	Medical subject headings
MDR	Multi-drug-resistance
MHC-I	Major histocomapatibility complex - I
MLD	Medial lethal dose
MOD	Ministry of defence
MOF	Multiple organ failure
MOH	Ministry of health
MOI	Multiplicity of infection
NCB	National center of biotechnology
NICD	National institute of communicable diseases
NIIEG	National research institute of epidemiology and hygiene
NO	Nitric oxide
OPS	O-Polysaccahride
PCR	Polymerase chain reaction
PLC	Phospholipase C
RKI	Robert Koch Institute
s.c.	Subcutaneous
SRCAM	State Research Center for Applied Microbiology
T_H1	T helper cell type I
T_H2	T helper cell type II
TLR-2	Toll-like receptor-2
TNFα	Tumor necrosis factor-alpha
TTSS	Type III Secretion System
UNICEF	United Nations Children's Fund
USP	United States (Pharmacopaeia) Plague vaccine

USSR	United Soviet Socialist Republic
VEE	Venezuelan Equine Encephalomyelitis
WHO	World Health Organization
WWI	World War I
WWII	World War II
Ysc	Yersinia secretory proteins
YOPs	Yersinia outer membrane proteins

1 INTRODUCTION

As the 21st century unfolds, infectious diseases remain one of the most significant threats to our economy, our food animal production systems, animal welfare, and most importantly, the lives of people worldwide, regardless of their economic standing (WHO. 1970, 1995, 2000, 2004). The potential use of biological threat agents for bioterrorism or bio-warfare further undermines the security of our society (CDC. 2000, KONDIRK et al. 2003, TITBALL and WILLIMASON 2001, 2004).

While the historical records on using biological agents' for offensive purpose in human history goes back to centuries, the intensity of state sponsored research activities to use biological products and microbes as weapons in warfare began as the world was ideo-politicaly divided in to East-West blocks commonly know us cold war era (CHRISTOPHER et al. 1997, CNS. 2005, CBW. 1998). Among others, the causative agent of plague (*Y. pestis*), Glanders (B. *Mallei*) and Melioidosis (*B. pseudomallei*) belongs to the most intensively studied bacterial pathogens of biological weapon (BW) interest in both blocks (ALIBEK. and HADELMAN 1999, DOMARADISKIJ and OREINT 2006). Their ubiquitous characteristics, availability, capability of aerosol transmission particularly (*Y. pestis*), the absences of vaccines and stringent control system makes them attractive candidates to some of those who has a willingness to use them as BW or bioterrorism. Now we live in an area of fear, in which we are faced with different types and degrees of insecurity. Nobody knows from where the danger might come.

There can be no doubt as to the fact that the ideological East-West conflict was one of the central problems of the globe of the last century, a period of tension, driven by a complex interplay of ideological, political, and economical factors contradictory to one another, which led to shifts between cautious cooperation and often better superpower rivalry over decades. The foundation was the bipolar military competition between the two superpowers the USA and the USSR and their respective allies and developing countries, thus this era was more portrayed as an era of "proxy war" because of its global impact on the rest parts of the world (COWDREY. 1984, CAUDLE III. 2007, BEECHING et al. 2002, ONISHENKO. 2004).

Due to the relatively independence of the East and West blocks and the closed character of the Soviet ideology, there was little reason to speak of any influence of the one on the other. Therefore, research activities, for offensive or defensive purposes were mainly, conducted under military auspices with high degree of secrecy and controversial testing programs independently. Several countries began programs to develop BWs following World War I (WWI) including Britain, Belgium, Canada, France, Holland, Italy, Soviet Union and Poland (BELLAMY and FREEDMANN 2001). Although biological warfare was the subject of detailed examination pursuing WWI, However, the most infamous biological weapon program (BWP) was probably that begun by Japan in 1932 in occupied Manchuria (HARRIS. 2001) that changed the scopes of research activities on highly contagious diseases of military importance dramatically. Thus, establishments of military research centres, for aggressive, offensive, and defensive research developments began under high

secrecy in both East and West blocs (KLIETMANN et al. 2001, GEISSLER. 1998, FRISCHKNECHT. 2003). Major research and development programs first emerged in the late 1930s and early 1940s. For instance, the development of biological weapons program which involved an extensive lists of causative agents of Plague, Glanders, Melioidosis, Anthrax, Tularemia e.t.c., at Camp Detrick, Frederick, Maryland USA as the headquarters of the arm's BW research was implemented in 1943, with approximately 3,800 military and 100 civil personnel. Where many of the efforts involved military researchers, other from Public Health Service, other Federal Agencies and Civilian Research Institutions were also involved (BERSTEIN. 1987, SANDRA. 2000).

During the two decades following the Second World War, laboratories, inclusion of viral, bacteria, and toxic mycosis that cause diseases in animals and plants were intensively studied (FRANZ et al. 2007). Historical records shows that USA, UK, Canada and the former Soviet Union (FSU) justified the importance of their program, because they have learned that since 1937, Japan had conducted a large biological warfare program, including human test, at its Unit 731 in Manchuria (HARRIS. 1997), the chemical warfare used the execution of bacterial warfare, in this case *B. mallei* by Germans, in the WWI, with series consequences on animals like Horses and Mules (GEISSER. 1999, WHEELIS. 1998, REDOMOND. 1998). The German army was considered, to be the first to use weapons of mass destruction, both biological and chemical agents, during WWI followed by Italian (FRISCHKNECHT. 2003). The Second World War has also shown similar inputs in FSU as to the West after the conquest of Red Army against Japan in Manchuria (DOMARADISKJI and ORENT 2006).

Although, all military research establishments, in general are covert, the scale of secrecy, intensity of research, span of time, area of competence and precedence in specific agents of BWPs interests like plague, glanders were much different. In the FSU research activities of these level were planned and controlled by state and kept under high secrecy till the collapses' of the Soviet Union. In most case, it is incomparable to that of the research approaches known in Western hemisphere (PEARSON and LEE 1985, LESKOV. 1993). For instance, the existence of large-scale production and storage capabilities of Plague agent (*Y. pestis)* and genetically manipulated agent of glanders (*B. mallei)*, to the level of high resistance to chemotherapy and vaccines, among other BW agents were some of the factors that distinguishes the Soviet Programs or in area of defence, the development of live plague vaccine based on EV76 line NIIEG used for more than 7 decades and continued to be used in some member states of FSU (ALIBEK. 1999, HENDERSON. 1999, ZLINISKAS. 2006).

Although state sponsored and institutionalized research activities on plague microbes goes back to the Czar period, Its main role were registration of natural foci, controlling endemic outbreaks like Typhus, Cholera, Glanders, and exotic diseases like Melioidosis e.t.c., prevention and prophylaxis measures. It was in 1928 that the Red Army Military Council made a decision of changes of the scopes and directions of the establishments from defensive to offensive research activities, recruiting specialized personnel's (NARKEVICH et al. 1989, 1991).
Y. pestis, *B. mallei* and *B. pseudomallei* were included in the program of all BW research activities known in both blocks. According to the Centers for Disease Control and Prevention (CDC), ranking

system, *Y. pestis* belongs to category A, similar to the Russian ranking system and *B. mallei* and *B. pseudomallei* to category B (ROTZ. 2002, CDC. 2000). On the contrary, in Russian ranking System both pathogenic *Burkholderia* species are listed in the second row of the last half six pathogens of category A of agents of great concern of potential uses of bioterrorism (KONDRIK et al. 2003).

It was estimated that *Y. pestis* alone killed over 200 millions of humans throughout history and still remains endemic in certain area of the world. Annually over 1000 cases of plague are registered by WHO (WHO. 1970, 2000, INGESLSBY. 2000, CASHMAN and BALDOR 2001). *Y. pestis* has several characteristics, like potential for high mortality of aerosol infection, ready dissemination from person to person, availability and the capacity to cause public panic, which makes it attractive to bioterrorism, and may be used to target humans (SORVILO et al. 2005). So far, there is no vaccine, no stringent control measures, no prompt and precise methods of diagnosis and effective treatment, that presents us with a dilemma now that enforce the development of vaccine against plague agent.

The development of vaccines and immunization is one of the most important medical innovation in public health care system. It is acknowledged by the World Health Organization as being 'among the most cost-effective of health investments'. So far vaccination has reduced morbidity and mortality from infectious disease more than any other medical intervention. It was a triumph of global immunization programmers that enabled the global eradication of Smallpox which was final reported by the Global Commission of World Health Organization that Smallpox eradication was certified in 1980 Geneva, Switzerland. However there are a number of infection diseases of human and animals for which no vaccine is developed yet, like glanders and melioidosis or commercially no more available, like in case of plague for human use worldwide save Russia and some member states of FSU (FEODEROVA et al. 2007, 2008).

1.1 Peculiarities of the Soviet Microbial Science

The unique characteristics of Soviet microbial science among others can be grouped in: 1) Unique establishments - The establishment of the Anti-Plague Institutes (API) in 1920 and 50 years later, the "Biopreparat complex" comprised of 52 sites that provide extensive research, development and production capacity. Highly qualified scientists and technicians estimated to more than 50,000 were employed in the last three decades of Soviet Union. (KLEITMANN. 2001, ONISCHENKO. 2004). During this period the Soviet BW program not only caught up the US BWP, but it became the most sophisticated BWP in the world by far. Substantially increased in size and scope. In contrast, in this period offensive research activities on BWP in Western hemisphere, like in UK in 1950th and USA in 1969 were stopped. Biopreparat complex was closed for foreigners, and what is known about it in Western hemisphere first, come from the senior defectors from FSU to the West. If the information published by senior defectors, is steadfast one can say the Biopreparat complex was/is a gigantic factory of new gene manipulated infectious diseases, which is still secret (ALIBEK. 1999).

The API network operated in the whole region of the FSU for more than a century, consequently. It has high competence and research experiences of the pathogens in concerns and other highly infectious diseases endemic in the region or exotic pathogens and training experts (NARKEVICH et al. 1991, ZILINSKAS et al. 2006). No comparable research establishment and networks of the type, exists world wide, 2) Research intensity and priorities - Although a number of states were engaged in studying these three pathogens for offensive as well as defensive purposes, the significant differences between the western research approaches and in the FSU was that research activities in FSU were intensive, continuous, and the causative agents of plague, glanders and melioidosis belongs to the prior groups of research activities, whereas in the Western hemisphere, research activities on these agents were sporadic, with decades of gaps and discontinuities, 3) Availability of the pathogens and diversity - In the FSU/CIS, the pathogens in concern, except the causative agent of melioidosis are endemic. About 216 millions of hectares (aprox.10 %) of the territories of the FSU consists of natural foci with constant and recurring epizootic activities of plague and high frequency of intraspecific diversified strains of plague microbe are circulating in the region. These strains are unique to the natural foci of the region and are not known outside FSU /CIS (ANISIMOV et al. 2004), 4) Differences in Taxonomy:-The Russian API has its own taxonomy of plague microbe that differs from what is known in International Code of Nomenclature of Bacteria (ICNB) of *Yersina pestis* taxonomy predominately used in West, which is based on the classifications of very limited biochemical characteristics in to three bovars (Antiqua, Medivalis and Oreintalis) (NIKOLAEV. 1972, SLUDISKI. 1998, DEVIGNAT.1951). Wheras, in the FSU/CIS, the in 1972, standardized taxonomy of *Y. pestis* based on numerical analysis of 60 phenotypic features is currently used in research activities (TIMOFEEVA. 1972, ANISIMOV et al. 2004,).

It is also worthy to note some remarks one at a time to realize the significance of FSU/CIS research achievements from the western point of view on this particular subject. For instance, the most popularly read *Scientist* journal edited by GINSBURG. (2005) published on plague an article titled as (to fight plague, look to the Russian's past). Another prominent microbiologist and bioweapon experts, ZILINSKAS. (2002) commented, once after having some clues from the FSU/CIS experts said, "On the biological side we are far behind, there is a whole history of things we on this planet we don't even know about." That indicates the significance of research activity of FSU/CIS and comprehension to understand the plague pathogen initially and for the development of efficient vaccine against plague in the future.

A recent reported cases of glanders outbreak in horses from autonomous region of Chita located in South Eastern Siberia in Russia, on the border with China and Mongolia, where more than 100 infected and positive tested horses were killed indicates the endemicity of *B. mallei* in the region (PROMED. 2007), 5) Area of concentration on the development of vaccines and prophylaxis - Since 1936, experts in FSU/CIS predominantly concentrated in studies and development of live plague vaccine based on *Y. pestis* EV 76 strain proposed by Girard, and *Y. pestis* EV 76 line NIIEG is the most common derivative strain for human vaccine development. The vaccine was known for its prompt, high induction of plague immunity, stability, and effective after administration by different routes , including, subcutaneous (s.c.), cutaneous, inhalation, and oral applications (SALTYKOVA

and FAIBICH 1975, ANISIMOV et al. 2004, FEODOREVA et al. 2007,). The immunological load induced by live dry vaccine in human revealed the higher blood levels of all T lymphocyte population on 14 day after vaccination (BOGACHEVA et al. 2009). Currently new candidate strains of *Y. pestis* are under investigation to develop an improved vaccine based on parental strain of EV76 line NIIEG and its mutant known as *Y. pestis Δ lpxM,* one of the promising candidate of forthcoming live attenuated plague vaccine development in Russia (FEODOREVA et al. 2007,2008)

In the Western hemisphere, series of whole killed cell vaccine based on different *Y. pestis* strains (USP), were developed. The vaccine based on *Y .pestis* 195/P strain was the mostly used killed plague vaccine in human, mainly in USA . The vaccine is no more available for human use(MEYER. 1974,WILLIASON. 2001). For the last two decades vaccine research activities in Western hemisphere concentrates on recombinant protein expression technology, investigation of single or recombined immunogenic antigens of plague microbe, such as purified Capsule (F1) antigen or purified V-antigen. The. Sub-unit vaccine (F1-V), a fusion protein composed of both proteins or separately purified F1 and V antigen (Recombinant vaccine) are expected to be licenced in near future (WILLIASON. 1995, 2009, TITBALL and WILIAMSON 2001, 2004).

5) Major records known about plague pathogen - Today the major records mainly known about plague, some on glanders and melioidosis are not available in English language publications. They are written in Russian language, published in Russian scientific journals, predominantly and most of them are rarely known in researches published in English language. In cases where they are cited, misunderstandings, or incorrectness are frequent. For instance, plague vaccine research review published by JEFFERSON et al. (2008), the cochrane collabration® titled “Vaccines for preventing plague (Review)”.

The Author says under the subtitle of vaccines; 1) Aerosol vaccines (from USSR state manufactures), “This is a live attenuated avirulent vaccine, produced in many centers in the former USSR, which has been in use since 1908 […]. For example live attenuated vaccines are thought to be less effective but have a lower incidence of side effects […] EV76 can be administered as an aerosol or subcutaneous.” The statement that says, “Live attenuated vaccines are thought to be less effective, but have lower incidence of side effects” is incorrect! It is known for long period that live attenuated plague vaccines provided a high degree of protection in comparison to vaccines based on whole killed cell, like Haffkine, or USP vaccine series (GIRARD and ROBIC 1936,1963, MEYER.1970,SALTYKOVA and FAIBICH 1975, RUSSEL et al.1995), If that was the case as the Author argues, that killed plague vaccines provided more protection as live vaccines, then all known killed vaccines beginning from Haffikine vaccine to a series of USP vaccine could have been not discontinued from production and commercially no more available for human use and the live dry plague EV 76 line NIIEG could have never been used for more than 7 decades with its international trade name *Vaccinum pestosum vivum siccum*® produced in Russia and continued to be used in the countries of FSU (FEDEROVA. 2007, 2008).

Although the first experimental studies on humans using live attenuated plague vaccine was designated "Pest avirulent" by STRONG. (1908), as there is no evidence at this period that the vaccine strain was EV76. What is know is that the virulent *Y. pestis* EV 76 strain was initially

isolated in 1926 by Girard and Robic from human case of bubonic plague, Madagascar, attenuated by multiple passages in vitro and proposed to be used as vaccine by Girard 1932, where EV stands for the initial name of an European child died of plague (GIRARD. 1936, 1963). This strain was distributed to various laboratories in the world and there are consistent evidences that showed, it can yield several local and systemic reactions, similar to infections induced by wild type, that differs from one to other laboratories which makes impossible to be licenced by the neutral bodies like WHO in the past.. Studies in FSU specifically on the EV 76 strain in 1936, in NIIEG, Kirov, API 'Microbe', Saratov, Russia not in 1908 (SALYTKOVA and FAIBICH 1975, FEODEROVA et al. 2007,2008). It is consistent that live attenuated plague vaccine is known for its efficacy of protection, do provide protection against bubonic and pneumonic plague, there is no evidence that killed plague vaccine do provide protection against pulmonary infection, but contracted pneumonic plague. It is also consistent that live attenuated plague vaccine does provoke both arms of the immune system better and consists of all required antigens to induce the immune system the the host, which are critical for sterile-immunization against plague microbe. In general, there are more inaccurate views on live plague vaccine of (EV 76 line NEIIG) in English language published when cited , because it has neither been used, studied nor licenced outside FSU/CIS.
The main concern of live attenuated vaccine of plague known is its reactogenicity, safety, public acceptance and the fear that it might reverts from avirulence to virulence "revertants" (MEYER. 1970, 1974, RUSSEL. 1995, FEODOREVA et al. 2007), but not that live attenuated plague vaccines are less effective than killed plague vaccines. Furthermore there are a number of evidence that confirms that the parental vaccine strain EV 76 of line NEIIG showed no signs of alterations in its morphological, biochemical characteristics or immunological value studied after three decades of storage (SALTYKOVA and FAIBICH et al. 1975).

Recent evidences showed by KUTYREV VV et al .(2009) who compared several genetic characteristics of the vaccine strain EV and its putative "virulent derivatives" obtained after passages through highly susceptible animals in order to identify the strains- “revertants” and establish their possible origin using PCR and DNA-DNA hybridization allowed to establish that virulent "revertants" are not derivatives of the EV vaccine strain, because they do not belong to East biovar, do not have ribotype characteristic for EV strain and contain pigmentation area, which is absent in vaccine strain, an evidence against possibility of reversion of vaccine EV strain to virulent form in organisms of highly susceptible animals, that confirm its stability and safety for vaccination.

The API has a long period of surveillance, unique research records, consistent monitoring activities of the natural foci and controlling plague outbreaks and prevention that goes back to 1920th The API controlled deadly endemic diseases and prevented the importation of exotic pathogens from other countries that could threaten human populations, livestock, and crops in the FSU. It is also one of the known achievements of the FSU in its field and Public health services in combating disease outbreaks and prevention (NARKEVICH et al. 1991, DOMARADISKIJ and ORENT 2006, ZILINSKAS et al. 2006). In area of immunology, consistent research and application of live attenuated plague vaccine for more than 7 decades. The development of different forms of plague

vaccines like non cellular or synthetic, vaccines also known as “chemical vaccine” proposed to be used as booster vaccine (DALVADYANTS. 1997), or an “aerosol vaccine” in combating disease outbreaks in case of Bio-attack or Bioterrorism (ALEXANDROV. 1962). These are some of unique achievements of FSU, for which no comparable studies are available in Western hemisphere.

Although the research activities of FSU is known as “The enclosed world of secret microbial science.” in the past. Today, lack of understanding and integrating the novel achievement, know-how, resources available to the rest part of the world might led to a bottle-neck for forthcoming vaccine development particularly, against plague agent that provides universal protection, have global acceptability and global impact.

The current research activities on pathogenic Burkholderia species concentrates on searching for immunogenic fragments, virulence determinant factors and mechanisms of pathogenesis. Although recent advances have increased in understanding the infection, protective immunity is sorely lacking (HARALAND. 2007).

In FSU/CIS, although varies conventional methods of vaccine development, like vaccine based on killed cells, Homologous vaccine, Hetrologous vaccines or Recombinant antigens based on other intracellular pathogens were conducted in different species of experimental animals, successfully clearup of the organisms from the host is not yet obtained (MANZENYUK et al. 1999. ILYUKHIN et al 1999,2002) Depending on the species of experimental animals used some forms of vaccine showed some low level of protection against virulent Burkholderia infection. Furthermore, There are some proposal of the development of vaccine against pathogenic Burkoholderia species based on fragments inserted to *F. tularensis* (ILYUKHIN et al. 1999), vaccine based on avirulent strain of *B*. *thilandensis* as a prototype in developing new vaccines or vaccine based on surface antigens fragments (ILYUKHIN. 2002). Currently there is no vaccine commercially available against agents that cause glanders and melioidosis either in clinical trials or licenced form worldwide.

Today more than ever, to fight this kind of war, to develop vaccines against pathogens of BW interest is not only the duty of a few researchers or institutions, single, two or three states as it is now, rather it needs global approaches for finding global solution.

To better understand the framework of the dissertation, historical backgrounds of the development of API, its exceptionality, backgrounds of the establishments and its scientific research publications, area of competence which are relevant for this work are thoroughly analysed.

It is important to understand where the word “plague” was used broadly to refer to infectious diseases causing high morbidity and mortality, where by plague caused by *Y. pestis* was /is endemic in the region, and belongs to the first line of pathogen to be fought by the API. Thus, the main core of the work concentrates on research publications on vaccines against plague, glanders and melioidosis diseases written in Russian language and published in Russian scientific

literatures in comparison English language publications as the subject coincides. For some unique research developments and results for which there are no comparable studies known in English language publications, analyses were made according to their relevance to the topic in description and future perspectives in developing new or improved vaccines based on the current standard of global knowledge. It also endeavours in evaluation of the delicate balances of major immunogenic versus virulent antigens which can be included in the development of newer or improved vaccine based on the current state of global knowledge on an antigen as single or recombined form, level of protection, safety and efficacy required for a new vaccine development in future.

Live plague vaccine of *Y. pestis* 76 EV derivative of NIIEG line the most known plague vaccine strain studied in depth in FSU and now in Russian federation. Thus, this work also thoroughly analyses the background of its development, its role in the region and its drawbacks for licencing in global level, and impact in future developments of new or improved vaccine against plague.

Problems that are considerably important in developing vaccines, like phenotypic and genotypic diversity, their scopes of multidrug resistance, and unique characteristics of the pathogens that makes them ideal candidates of vulnerability, availability of the pathogens are taken into consideration.

Multidrug resistance is one of the challenging factors in Public Health sector today. Although plague microbe is described to be sensitive to first class antibiotics some strains that were isolated from a patient died of pneumonic plague and from patients living in natural foci showed high resistance to antibiotic (GALIMAND. 1997, EVCHENKO et al. 1997). A dozen of antibiotics sensitivity studies done in vitro against a number of Yersinia strains isolated from different natural foci showed no efficient protection in experimental animals was reported (SAMOKHODKINA. 1997). That indicates a wide spread of antibiotic resistance strains of the pathogen and the requirement of developing a vaccine as the appropriate steps to the solution.

The alarming spread of antibiotic resistance in *B. pseudomallei*, exhibits resistance to diverse groups of antibiotic including third generation cepahlosporins, penicllins, rifamycins and aminoglycosides (HSUEH et al. 2001), resistance to quinolones and macrolids more over limits the therapeutic options of melioidosis (CHENG and CURRIE 2005). Furthermore, the organism is known for its slow responds to most antibiotics and common relapses after apparently successful primary treatment, some expertise call it “silent killer” (CHAOWAGUL et al. 1993), that also certainly need vaccine development. Because of its long latent period, melioidosis is known as Vietnam's time bomb in USA.

In comparison to human melioidosis, there are very few scientific literatures describing the susceptibility of *B. mallei* to antibiotics in human infection post antibiotic era. It is believed to have similar characteristics to the very closely related *B. pseudomallei* species. One recent reported case of laboratory-acquired human infection was a patient who worked with agent. Treatment with

cephalexin and cetriaxone did not improve the situation of the patient (SRINIAVASAN. 2001).This indicates that *B. mallei* is capable to relapse despite completing the therapy, that also shows the requirements of development of vaccine as the most appropriate solution.

Thus, as a consequence of these major differences of research approaches, experiences with the pathogens in concern and the development of vaccines apparently may different correspondingly between West and CIS, exact Russia, that consequently may lead to a limited acceptance of one form of a vaccine by the other in licencing for global effectiveness as it was in the case of plague vaccine in the 20th century such as by a neutral body like WHO.

2 CHALLENGING CHARACTERISTICS OF THE AGENTS

2.1 Yersinia pestis

The genus *Yersina* a member of the family *Enterobacteriace* consists of 14 species, of which 3 are human pathogens, *Yersina pestis*, the causative agent of the systemic invasive infectious disease classically referred to as a plague (DSMZ. 2008, PERRY and FETHERSTON 1997), and the enteric food-and water-borne pathogens *Y. pseudotuberculosis* and *Y. entercolitica* (BERCOVIER et al. 1980, MURRAY et al. 1995).

Yersinia pestis is a gram-negative facultative anaerobe, non-spore-forming, non-motile coccobacillus (0,5 to 8 µm in a diameter and 1 to 3 µm long), that exhibits bipolar ("closed safety pin") staining with Giemsa, Wright's or Wayson staining (BAHMANYAR and CAVANAUGH 1976, WHO. 2000, CIDRAP. 2003). *Y. pestis* is not a fastidious bacterium; It grows well on blood and many other enteric media. The organism grows at temperatures from 4 up to ~40 °C (optimum at 28 to 30 °C); the optimum pH for growth ranges from 7.2 to 7.6; however, extreme of pH 5 to 9.6 are tolerated. It is very sensitive to high temperature, drought. At the temperature of 50 °C the organism dies within 30-40 minutes, at 70 °C, within 10 min., by boiling in 1 min. Direct sunlight kills the organism within 2 to 3 hrs. It is preserved by low temperature. It can survive for 396 days under temperature ranges from 0 °C-15 °C in fleas, in mites and ticks up to 509 days. It's a weak concurrent against the coexisting microflora around it (BRUBAKER. 1972, PERRY. 1997, SLUSARI et al. 1997). The L-form of *Y. pestis* could persist in host animals and in carriers such as mites more long time (Years) (ZYKIN et al. 1989). Enzootic circulation of *Y. pestis* in natural plague foci requires active infection of host rodents and grows in the fleas, which is essential for the maintenance of endemic infection in natural foci (PERRY. 2003).

Y. pestis harbors at least three plasmids one of which is common to the entero-pathogenic species *Y. pseudotuberculosis* and *Y. entercolitica* (FERBER et al. 1981, PORTNOY et al. 1985). The other two plasmids designated pMT1, mostly known as pFra and pPCP1 are unique to *Y. pestis* (BRUBAKER. 1991). Plasminogen activator *(pla)*, the bacteriocin pesticin *(pst)*, and a pesticin immunity protein *(pim)* are encoded on the 9,5 kb pPCP1. The large 100 kb to 110 kbp plasmid encodes two potential virulence determinants factors: the Murine Toxin *(Ymt)* and the Fraction1 (F1) capsule antigen (CHERPANOV et al. 1991, BEN-GURION and SHAFERMANN 1981, FILIPPOV et al. 1990, KUTYREV et al. 1986)

Yet much what is known in English language publications about the genetic and phenotypic properties of *Y. pestis* comes from studies of a limited number of strains commonly found in Americas, where there is very restricted genetic diversity (RADNEDGEL et al. 2002, ANISIMOV et al. 2004). Thus much of the pathogenic potential of *Y. pestis* for humans remains largely unknown, many of which are found in isolated region of FSU and Asia and not easily accessible to researchers.

What is known about the pathogen in this region is predominately written in Russian language and Russian scientific journals (TIMOFEEVA.1972, KOKUSHKIN.1994, ANISIMOV et al. 2004).

The first compact summary of Intraspecific diversity of *Y. pestis* known in FSU and its significance compared to strains known in the Western hemisphere was published in English language by ANISIMOV et al. (2004). Not only that about 10 % of the territory of FSU comprises natural plague foci, with its constant and recurring epizootic activities but, also the most diversified, dynamic strains of plague pathogen are circulating in the region. A number of strains isolated from the region are known to have a high intraspecific diversity, some of them lacks one or two of the plasmids *Y. pestis* harbors and some of them consists additional cryptic plasmids of different molecular masses, whose role is not yet elucidated are know and are equally virulent in experimental animals when compared to the wild types (NARKEVICH et al. 1991),

The Russian museum of microbial collection, Institutes and laboratories possess the most highly diverse strains of plague bacilli in quantity and quality in the world. Suggesting, that understanding these atypical strains only is even more important, because for one they are rarely known, further more there are no specific diagnosis methods and may take an advantage of bioterrorism utilization. Thus, It is essential to include in research activities in processes of developing vaccine against plague of universal protection and acceptance.

Being one of the oldest identifiable diseases known to man, plague caused by *Y. pestis* remains enzootic and endemic in many natural foci around the world (AMPEl. 1991, WHO. 2000). Until now, it is not known what “wakes” them up and leads to periodical outbreaks, so it is possible to expect different surprises in a form of swarms of epizootics of plague. What makes the matter even worse is that, it is not known from where the danger may erupt if no permanent and stringent surveillance is in place (STEPHEN. 1995). One of the most disturbing side-effects of modern life is the speed with which diseases can spread around the globe. Decades ago, it was much less likely that an outbreak of infectious diseases occurring on one continent could cross-oceans to another. Intercontinental air travel now makes it possible for a disease to arrive in other continents before signs of the original outbreak have been recognized. The World Health Assembly attributes the problem of new emerging or re-emerging diseases such as plague, to the following trends: With the increasing global population many are forced to live under conditions of overcrowding, inadequate housing, and poor hygiene; more frequent international travel leads to rapid global exchange of human pathogens; changes in health technology, food production, as well as its distribution (including international trade and handling), climate changes, natural catastrophes, immigrations of vectors also known as “amplifiers”, create new opportunity for pathogens; and human behavioural changes expose large segments of the global population to disease not previously experienced; expanding areas of human habitation expose thousands of people to enzootic pathogens previously unknown as causes of human disease; and microbes continue to evolve and adapt to their environment, leading to the appearance of new pathogens (WHO. 1995).

Furthermore, there is a fear that science of microbiology, genetics could be misused. A fear, that microbiological agents can be used as weapons of mass destruction. The belief that the anthrax attacks in the year 2001 were carried out by a scientist and that the bacteria came from a US laboratory has made microbiologists suspect as well as potential saviours (ATLAS. 2003, CDC. 2001a,2001b). Recently, *The Washington Post* (2010) reported:*"The Justice Department officially ended its eight-year investigation of the 2001 anthrax attacks Friday with the release of hundreds of pages of documents that starkly portray the mental unravelling of the deceased Army scientist accused of committing the worst act of bioterrorism in U.S. history*." That demonstrated the new perils facing the world.

It must be also emphasized that it was the distinguished scientific community who initiated the movements to create biological, chemical and nuclear weapons, sponsored by ambitious leaders. *Y. pestis* is classified as level A biothreat agent, characterised as high-priority agent, easily to disseminate, with a high mortality rate, with the capacity to cause panic and social disruption and requiring specialized public-health responses (INGELSBY et al. 2000, ROTZ et al. 2002, CDC. 2000).

Plague diagnosis may be confused with a number of other infectious diseases depending on the clinical manifestation (NEUBAUER et al. 2000). Bubonic plague may be confused with infections like streptococcal or staphylococcal lymphadenitis, infectious mononucleosis, tularemia, mycobacterial infection, e.t.c, (HULL et al. 1986, CROOK and TEMPEST 1992). Septicemic plague may be confused with meningo-coccemia and septicemia caused by other gram-negative bacteria. Pneumonic plague may be confused with other causes of acute severe community-acquired pneumonia, such as pneumoccal, streptococcus, *Haemophilus influenzae* infection, anthrax, tularemia, legionellosis and viral infections (POLITZER. 1954, HULL. 1987, MARGOLIS et al. 2008).

2.1.1 Differences in Taxonomy and its impact on Vaccine Development

The currently widely used classification of *Y. pestis* strains in the Western hemisphere is based on minor phenotypic and biochemical differences: biovar Antiqua reduces nitrate and ferment glycerol whereas biovar Medievals does not reduce nitrate and biovar Orientalis does not ferment glycerol (DEVIGNAT. 1951, TUMANSKII. 1957). The fact that *Y. pestis* is essentially a recently introduced pathogen into the Americans, 1900-1904, San Francisco, USA, indicates that the genetic and phenotypic diversity of isolates is relatively restricted, particularly compared to those from Central and East Asia, where distinct strains are identified in the respective niches

In FSU/CIS the classical subdivision is not directly used as a pre-eminent way of classification of plague microbe, because of some drawbacks, for instance, the biovar characterizations are not stable and one strain can undergo spontaneous phenotypic variation which would cause it to be classified into another biovar (INNOKENTEV. 1969, KOZOLOV. 1979). Strains of different biochemical, cultural morphological and virulence characters were realized permanently, even within a single species of hosts, which indicates, the plague microbe can maintain broad changes of spectrum (MAIKANOV. 1997).

The classification of the natural plague foci in FSU were primarily performed based on their geographical distribution and then based on primarily infected hosts found in each focus. Various strains of *Y. pestis* isolated from the territory of FSU and Mongolia were classified into "subspecies" *Yersinia pestis* subsp. *pestis* (sometimes referred to as the "main" subspecies) *Y. pestis* subsp. *altaica, Y. pestis* subsp. *caucasica, Y. pestis* subsp. *Hissarica, Y. pestis* subsp. *ulegecia* based on the numerical analysis of 60 phenotypic features that was standardized in a conference of the experts of the APIs of the Soviet Union, Ministry of Health, Ministry of Defence and other governmental regulatory agents , Saratov, 1985 resolution. These Taxonomy and Nomenclatures are currently used in the works of APIs in Russia and member states of FSU (APARIN et al. 1987). Chinese plague experts use their own classification utilizing the term "*ecotype*" for different *Y. pestis* subspecies. Groups differ in some phenotypic properties from Russian "*nonmain subspecies*" (TAN et al. 2002). However, these systems are not currently included in the International Code of Nomenclature of Bacteria (ICNB) at all. Diversity in genotypes and phenotypes is found even among plague isolates from the same natural focus. Thus, no system of classification is likely perfect having nearly 100 % specificity. Atypical or engineered strains with a variety of unusual characteristics might reasonable be expected to be a cause of human infection accidentally or deliberately and is warranted to have an impact in treatment, diagnosis and vaccine production (ANISIMOV et al . 2004).

Y. pestis is closely related to the gastrointestinal pathogen *Y. pseudotuberculosis*, and it has been proposed that *Y. pestis* is a clone that evolved from *Y. pseudotuberculosis* probably serotype O.1b (SKURNIK et al. 2000) 1,500-20,000 years ago (ACHTMAN et al. 1999) by application of Multi-locus sequence type (MLST) of house keeping genes. Therefore, *Y. pestis* seems to have rapidly adapted from being a mammalian entero-pathogen widely found in the environment, to a blood-borne pathogen of mammals that is also able to parasitize insects and has limited capability for survival outside the hosts (PARKHILL et al. 2001). Nevertheless, the symptoms of the disease, routes of infection, and the scale of virulence, public health care and security caused by the two Yersiniae are dramatically different (BRUBAKER. 1991, PERRY and FETHERSTON 1997). The enteropathogenic Yersiniae must survive in soil and water and bypass the host gastrointestinal mucosa following ingestion, whereas *Y. pestis* remains within the closed and protected environment of its flea vector, thereby ensuring by intradermal injection, a route that requires extensive dissemination to achieve favoured visceral niches which support the bulk of replication in vivo (PERRY. 2003, HINNEBUSCH, 1997)

The most impressive genetic difference between *Y. pestis* and the entero-pathogenic species in this regard is the presence in most but not all strains of the former of two unique plasmids (FILIPPOV et al. 1990), pMT1 (69 to 110kb) encoding (murine toxin and F1) and pPCP1 (9,6kb) encoding (plasminogen activator) are important virulence factors (BEN-GURION and SHAFFERMAN. 1981, FERBER and BURBEKER 1981, FILIPPOV et al. 1995, KUTYREV. 1986) and some small regions of chromosomal DNA (PARKHILL et al. 2001, DENG et al. 2002). Another major difference between *Y. pestis* and the enteropathogenic Yersiniae is the presence of as many as 30 copies of an insertion

element termed *IS100,* both, within the chromosome and on all plasmids (PORTNOY and FALKOW 1981, RAKIN et al. 1996, BOBROV and FILIPPOV 1997). They also share many additional processes that promote disease as reflected by carriage of a common sites in which a large number of genes encoding virulence factors such as Yersinia outer proteins (Yop) and the Yop secretory system (Yops) are clustered, as well as silent regulatory and anti-inflammatory functions. The generic term "low-calcium response," or Lcr plasmid, has been applied to this plasmid regardless of its origin; it is specifically termed pCD in *Y. pestis pCad* or PIB in *Y. pesudotuberculosis* and pYV in *Y. enterocolitica* (CORNELIS. 1998, IRIARTE and CORNELIS 1996, PERRY. 1997).

Comparison of the genomic content of this organism to the six published sequences of *Y. pestis* and their *Y. pseudotuberculosis* ancestor, based on classical glycerol fermentation (+ve) and nitrate reduction (+ve) done, showed that *Y. pestis* Pestoides F is an isolate that belongs to the biovar antiqua. This strain is unusual in other characteristics such as the fact that it carries a non-consensus V antigen (LcrV) sequence, and that unlike other Pla(-) strains, Pestoides F retains virulence by the parenteral and aerosol routes. The chromosome of Pestoides F is 4,517,345 bp in size comprising some 3,936 predicted coding sequences, while its pCD and pMT plasmids are 71,507 bp and 137,010 bp in size respectively. Comparison of chromosome-associated genes in Pestoides F with those in the other sequenced *Y. pestis* strains reveals differences ranging from strain-specific rearrangements, insertions, deletions, single nucleotide polymorphisms, and a unique distribution of insertion sequences. There is a single approximately 7 kb unique region in the chromosome not found in any of the completed *Y. pestis* strains sequenced to date, but which is present in the *Y. pseudotuberculosis* ancestor (GARCIA et al. 2007). This indicates, that in post genomic era, the three frequently used forms of Taxonomy of *Y. pestis* biovars is aniquated, consequently shows the importance of updating the taxonomical classification of *Yersina pestis* based on current global knowledge of the organism and strains known from different natural foci worldwide, which are not yet included in the International Code of Nomenclature of Bacteria (ICNB).

Further more sequencing of the entire genome of *Y. pestis* Orientalis strain CO-92 (PARKHILL et al. 2001) and a Mediaevalis strain KIM+ (DENG et al. 2002) has showed that both strains shared > 98 % of the genome sequence. In contrast, the two recent sequenced strains of *Y. pestis*, known as Nepal516 (CHAIN et al. 2006) and *Y. pestis* Pestoides F (GARCIA et al. 2007) showed distinguished differences unlike the classical known biochemical and phlogentical division. *Y. pestis* Nepal516 strain, isolated from human in Nepal, which has been biochemically characterized to belong to biovar Antiqua compared to strain antiqua "classical" antique biovar demonstrated that, there are differences between the two biovar antiqua lineage. That indicates grouping *Y. pestis* strains based strictly on the classical definition of biovars (predicted upon two biochemical assay) does not accurately reflect the phylogenetic relationships within this species (CHAIN et al. 2006). *Y. pestis* Pestoides F, the first sequenced strain isolated from the former Soviet Union by GARCIA E et al. (2007) indicates, unlike the classical *Yersinia pestis* strains, members of an atypical group of *Y. pestis* from Central Asia, denominated *Y. pestis* subspecies *caucasica* (also known as one of several pestoides types), are distinguished by a number of characteristics including their ability to ferment

rhamnose and melibiose, their lack of the small plasmid encoding the plasminogen activator *(pla)* and pesticin *(pst)*, and their exceptionally large variants of the virulence plasmid pMT- encoding murine toxin and capsular antigen (DONG et al. 2001, KOKUSKIN et al. 1994, ANISIMOV. 2004, BALAKHONOV. 1991, GRAMOTINA and PROTSENKO 1994).

One important finding of the plague-experts engaged in investigating enzootic strains of *Y. pestis* in FSU is that in some populations the plasmid contents were know to be quite stable, such as strains of *Y. pestis* subsp. *Caucasica* isolated from Voles and their fleas in Leninakan and Presvan. Whereas in some other populations there were a considerable variation among strains, such as , strains isolated from rodents living in close proximity. Strains isolated from Zanzegur-Karabah and Dagestan highland natural foci was found that the *pPst* plasmid that carries the genes for pesticin-fibrinolysin-coagulase activities was missing (KYTREV et al. 1992). In Mongolia It was found that the palsmid content of 894 *Y. pestis* strains isolated from patients, wild mammals and arthropods were divisible in to three distinct populations of strains based on the differences in molecular masses of the plasmid the strains harbors (BALAKHONNOV et al.1991, ANISIMOV etal. 2004). In China, in Yunnan province 1020 strains were found to carry plasmids of nine different sizes (ZHAO et al. 1990). In Brazil, pareaba state, a cryptic plasmid of 14.9 MDa was isolated from 26 strains (LEAL et al. 1989). Thus a variation of high plasmid profile, diversity of *Y. pestis* populations found in different region of the world needs further studies in order to understand their role in general and for the developments of vaccine specifically. Thus it will be important to answer the following questions:-1) Does the mutation of one plasmid or two major plasmid leads to more virulence or avirulence in comparison to the classical strains that harbors all known three plasmids? 2) Which role does the additional cryptic plasmids, whose role has not been elucidated can have on vaccine development? 3) Does the forthcoming vaccine known as Sub-unit/Recombinant vaccine based on two major antigens F1-V or rF+rV respectively of *Y. pestis* provide efficient protections against these isolated from natural foci such as from FSU, Mongolia, China, Brazil or from other natural foci found globally which are yet known but may circulates in natural foci and are virulent?

2.1.2 Multidrug Resistance: Unreliability upon Antibiotics

As the majority of gram negative micro-organism, usually *Y. pestis* isolates are uniformly susceptible to antibiotics, but now new highly resistant strains to first class antibiotics have evolved, and are going on reproducing even faster (SMITH et al. 1995). Antimicrobial-drug resistance is an increasing important factor and poses a serious international challenge to public health in both communities and institutions (WEISE et al. 1998). In spite of the significant role of antibiotics in treating bubonic plague, but not pneumonic plague, new multi-drug resistant strains of plague bacilli were reported in vitro (FREAN. 1996) isolated from patients (RASOAMUNAN et al. 1995) and strains mostly isolated from Suslik's (*Citellus citellus*), habitats in natural plague foci in the CIS (EVCHENKO et al. 1997). Streptomycin, chloramphenicol, and tetracycline, are used to treat plague, and sulfonamides are recommended for prophylaxis (BARNES and QUAN 1992). The new isolate *Y. pestis* 19/95 biotype orientalis was isolated in the Ambalavo city of Madagascar from a 16

-year's old boy (RASOAMUNAN et al. 1995). It was highly resistant to chloramphenicol, kanamycin, streptomycin, sulfonamides, tetracycline, and minocyclin. The resistance genes were carried by a plasmid, pIP1202, approximately 150 kbps, that could conjugate to other *Y. pestis* isolates (GALIMAND et al. 1997, DENNIS. 1997) which demonstrates the risks of the spread of resistance in *Y. pestis*, a species previously considered universally susceptible to antibiotics. Resistance to ampicillin was due to the production of a beta-lactamase, and resistance to kanamycin was due to the synthesis of type I 3'-aminoglycoside phospho-transferase. The strain was also resistant to high level of streptomycin as a result of the production of 3'-9'-aminoglycoside adenyltransferase (GARROD and WORTH 1962). The second high-level resistant strain to streptomycin (the reference antibiotic for plague treatment) due to streptomycin phosphotrasferase activity was named *Y. pestis* 16/95 and isolated in the Ampitana town, Fianarantsoa province, Madagascar from a human case of bubonic plague, that is 120 km apart from Ambalavao city , where *Y. pestis* strain 19/95 was isolated (a typical strain of ribotype B). The new strain is described to be a Madagascar-specific ribotype Q. The resistance genes were carried by a plasmid pIP1203 that could conjugate at high frequencies to other *Y. pestis* (GUIYOULE et al. 2001). The new multi-drug resistance isolates reported by GALIMAND et al. (1997), raises several questions, which are not yet explained: 1) where the isolate did acquired its resistance plasmid? 2) do resistant enteric bacteria perhaps exists in the rats in the region, while rats mainly live in a fecal contaminated environment (BYRNE and SUSAN 1998), with multi-drug resistance human fecal flora? More questions are to be answered through systematic, domestic and global surveillance to find appropriate responses against multi-drug resistance in plague.

New strains of *Y. pestis* 19/95 biotype orientalis and *Y. pestis* 16/95, isolated from patients in Madagascar were reported to be resistance to multiple drugs and streptomycin respectively, which were carried by plasmids. That demonstrates the risks of the spread of resistance in plague bacilli, a species previously considered universally susceptible to antibiotics (GALIMAND. 1997, MCCORMICK. 1998, WONG et al. 2000, GUIYOULE et al. 2001, STEWARD et al. 2004).That indicates unreliability of treatment upon antibiotic therapy.

The results of studies done on antibiotic susceptibility of *Y. pestis* strains isolated in the years 1995-1996 from patients living in three natural foci found in the territory of the republic of Chechenia, also indicated that 23 % of the strains isolated from Pre-caspian natural foci were moderately resistant to streptomycin, amikacin, cefazolin, kanamycin, and polymixin. Additionally, 13 strains that belong to the Kavkazian sub-species isolated from the mountain regions of Dagestan, were found all, to be resistance to polymixin treatment, 23 % to streptomycin and amikacin, 61 % to kanamycin, 61.5 % of the isolates were not or weakly responding to ciprofloxacin and 53,8 % the isolates showed resistance to levomycin and cefalotin (EVCHENKO et al. 1997). In addition to the strains isolated from patients, resistance to antibiotics was also prevailed in strains isolated from nature in the region.

Another similar experimental study focused on antibiotic resistance of *Y. pestis* demonstrated, that an infection caused with non-capsule-forming ($F1^{-}$) strains and simultaneously treated with

tetracycline, ß-lactamin antibiotics, and quinoline with in the range of therapeutic dose, showed no effect. In contrast to infection caused by capsule forming ($F1^+$) strain of *Y. pestis* , which expressed an effective therapy (SAMOKHODKINA et al. 1997). In vitro investigation of the effectiveness of 14 antimicrobial agents investigated in 78 strains of *Y. pestis* showed that, antibiotics like streptomycin, tetracycline, chloramphenicol, azithromycin traditionally used for the treatment of plague were insufficient to kill the organism in all 78 strains (SMITH. 1995). Resistance of *Y. pestis* to antibiotic is also prevailed in macrophage cell culture, but not in nutrient medium (SAMOKHODKINA et al. 1997). A recent know case was reported from California, (MARGOLIS et al. 2008), where a 79–year old woman A treatment with ceftriaxone followed by scheduled ampicilin/sulbactam and levofloxacin, while the patient received a diagnosis of community–acquired pneumonia initially. As the diagnosis was changed to be a disease caused by *Pseudomonas aeruginosa*, the antibiotic regimen was also changed to meropenem and gentamicin. It was finally diagnosed that, it was a septicemic plague and the patient had received 10 day of effective *Y. pestis* covered and had an extended hospital stay, but she was died. MDR in *Yersina pestis* is not a question of if, but it is a question of when, that enforce vaccine development against a plague as the most appropriate solution.

2.1.3 Neither Stringent Control System nor Vaccine is currently available

The global availability of the plague pathogen, the capacity for mass production, aerosol dissemination, the high fatality rates of pneumonic plague and the potential for secondary spread of cases during epidemic, the rapid growing of MDR, the absence of prompt and precise diagnosis makes it one of the most serious concerns of ˙critical biological agents' of category A, that have a potential to be used as a biological weapon, which is difficulties to prevent (CDC. 2000, 2001a). Several characteristics of biological agents that may make them appealing to bioterrorism are, they are relatively inexpensive, readily available, easy to produce in quantity, effective in causing damage and confusion, easy to conceal and transport, poses delayed effect, significant economical, social impact and ability to cause panic internationally (HUGHES. 1999).

The epidemiology of plague following its use as a biological weapon would differ substantially from that of naturally occurring infection. Intentional dissemination of plague would most probably occur via an aerosol of *Y. pestis*, a mechanism that has been shown to produce disease in non-human primates and experimental animals (SPECK and WOLOCHOW 1957, WILLIAMSON et al. 2001. 2001 SMILEY. 2008). A pneumonic plague outbreak would result with symptoms initially resembling those of any other severe respiratory illness. The size of the outbreak would depend on factors including the quality of biological agent and characteristics of the strain, environmental conditions, and methods of aerosolization. The pathogenesis and the clinical manifestation of plague following an inhaled aerosolized *Y. pestis* bacillus would cause primary pneumonic plague which would be notified differently than naturally occurring plague. The incubation period to aerosolized plague has been found to be 1 to 6 days, in human and non-human primates and most often, two to four days (WU. 1926, SPECK et al. 1957, FINGOLD et al. 1968). The first sign of illness

would be expected to be fever, with cough, and dyspnoea sometimes with production of bloody, watery or less commonly purulent sputum and prominent GIT symptoms including nausea, vomiting and chest pain. Abdominal pain and diarrhoea might be present (CDC. 1997).

Currently there is neither stringent control system, nor vaccine is available in the Western hemisphere save Russia. Suppose a group of people are armed with it and would commit these acts in appropriate time and places, how do the world behave in this situation? Nobody knows. The only available vaccine is the live plague vaccine based on the strain of *Y. pestis* EV76 line NIIEG which has been used as human vaccine for more than seven decades and has continued to be used in the countries of of Former Soviet Union. Annually more than 100,000 people living and working in endemic region are immunized against plague (FEODOROVA et al. 2007, ISTC. 2008). It was never used and licenced in Western hemisphere.

2.1.4 Pandemics and Global Distribution

History has accounts of four, some groups it to three, plague pandemics; Egypt in 541 AD, Asian Minor in the 14th century, Europe in the 15th, and china in 1860, which were collectively responsible for the loss of 200 million human lives (WHO, 2000).

The *first pandemic*, known as Justinian's plague, occurred between AD 540-750, causing epidemics in Asia, Africa, and Europe. It is estimated to have caused nearly 100 million victims, and was followed by various smaller epidemics (DUPLAIX. 1988, MCEVEDY. 1988, WHO. 2000, BUCHRIESER et al. 2001).

The *second and third plague pandemic* is the well-known "Black death" began from Asia Minor in 14th century and spread to Europe from 15th-16th centuries. This pandemic was the beginning of a number of outbreaks of plague, which ravaged Europe and Africa in subsequent centuries, causing an estimated 50 million deaths, approximately half of them in Asia and Africa and the other half in Europe, where quarter of the population succumbed (MCEVEDY. 1988, SLACK. 1989). It entered Europe probably via the trans-Asian Silk Road during the early 14th century. In the year 1346, plague arrived in [K]Caffa (Modern Feodesya, Ukraine) at the Criema, It is widely believed as the results of biological warfare attack by Mongolian. The unwanted import landed in ports like Marseille and Genova and pushed inland. By 1348, plague had already entered Britain at Weymouth (BAYLISS. 1980). By the year 1352, 25 million people had died in Europe alone.

The *fourth pandemic* began in Canton and Hong Kong in 1860 and spread rapidly throughout the world, by rats aboard the swifter steamships that replaced slow-moving sailing vessels in merchant fleets. Alone, within 10 years (1894-1903) plague entered 77 ports on five continents. In India, were over 6 million deaths from 1898 to 1908. Case rate per 10,000 populations was registered in India 427; Senegal 269; Hongkong 244; Mauritius 227; Burma 122; Madagascar 116

and Uganda 88; to note some of the records of the extension of third pandemics of plague in the 20th century (WHO. 2000, DUPLAIX. 1988, CDC. 2005).

It was also in this period, precisely in 1894 that Alexandere J. E. Yersin and S. Kitasato independently discovered that *Y. pestis* causes plague. Three years latter W. Haffkine developed a heat-killed-vaccine and Simond confirmed that the rats were the vector of plague bacilli, over 200 species of wild rodents, found to harbor the bacilli. It was believed that, the reservoir of plague bacilli in the fleas of the Siberian marmot (tarbagans) was likely responsibly for the death of 50,000 men in the Manchurian pneumonic plague pandemic in the year 1910-1911. In the year 1900 the disease officially arrived in North America (RISSE. 1992). In the year 1914 plague arrived at the West-African coast, with an outbreak in Senegal, Dakar, where about 35,000 men died in 32 years. The giant rats, *Cricetomys gambiae*, which was abundant in the bushes, might be a responsible vector in Western Africa as tarbagans were in Manchurian (ECHENBERG. 2001).

Natural foci of plague are situated in all continents except Australia. It covers 6 to 7 % the dry land of the Earth, situated in a broad belt in the tropical and subtropical latitudes and the warmer parts of the temperate latitudes around the globe between the parallels of 55 degrees North and 40 degree South (fig. 1). However, with in there limits many areas are free of natural foci of plague, such as desert areas with few or no rodents, and the large areas of the continents forest particular in the tropics and high glacier-covered mountain. Hence, plague foci are not fixed, and if they can change in responses to the shift of the driving factors such as climate, landscape, rodent population migration, travel and transportation (DUPLAIX. 1988, PERRY and FETHERSTON 1997, WHO. 2000).

Cases of human plague have been known from time immemorial, and throughout history, some of the authors believe, that oriental rat flea (*Xenopsylla cheopis*) has been largely responsible for spreading bubonic plague (BAYLISS. 1980). In fact, plague is now considered as a re-emerging disease (SCHRAG et al. 1995) for at least three reasons; 1) there has been an increase in the number of cases reported to the World Health Organization, 2) plague re-appeared in an epidemic form in countries, including the Democratic Republic of Congo, Malawi, Mozambique, Zambia, Madagascar, India, USA, recently in where it had been silent for two to three decades, 3) the number of foci is gradually expanding in certain countries (WHO. 2000, 2003, 2006).

Plague is not an eradicated disease, a curiosity with importance only to medieval history (AMPEL. 1991). Indeed the official reactions of several countries show that plague remains one of the most feared of the infectious diseases. Outbreaks of plague in India in 1994 were notable because no cases had been reported in humans there for nearly 30 years. Notification caused global alarm, disrupted travel, trade, and resulted in severe economic repercussions with cost of two billion US dollars. They also highlighted the lack of preparedness of national and international health agencies to deal with plague epidemics. This zoonotic disease, with reservoirs on nearly every major continent, exhibits an impressive ability to overcome mammalian host defence (PERRY and FETHERSTON 1997, JOHN. 1994). Epidemiological studies in Surat showed that it was an outbreak

of pneumonic plague resulted from person-to-person respiratory exposure. The outbreak occurred in a setting of high frequency of severe fever. About 75 % of the suspected plague cased in Surat were young males. There were no confirmations of bubonic plague (DENNIS. 1994). The recent disease outbreak reported to WHO from Democratic Republic of Congo (WHO. 2006), where 100 cases of suspected plague including 16 deaths in Ituri district were pneumonic, Oriental Province, Ituri is known to be the most active "hot-spot" of human plague Worldwide. Recently, in USA a total of 13 human plague cases have be reported among residents of four states: New Mexico, Colorado, California and Texas (CDC. 2006). The WHO, estimates that there are approximately 3,000 cases of plague annually worldwide. The real is believed to be greater than what is registered by World Health Organization. In the year 2003 alone nine countries reported 2,118 cases and 182 deaths. 98.7 % of those cases and 98.7 % of those deaths were reported from Africa.

Today the distribution of plague coincides with the geographical distribution of its natural foci (WHO. 2006). Thus, the recent outbreaks of plague in India in the year 1994 remind us once again of the need to maintain a core of skills in infectious diseases and the public health infrastructure to detect, monitor, and combat a wide range of disease agents, some new, some revisiting and the importance of developing vaccines as the most appropriate option. Plague may have retreated over the past decades, but it has not gone away (WHO. 1994, DENNIS. 1994).

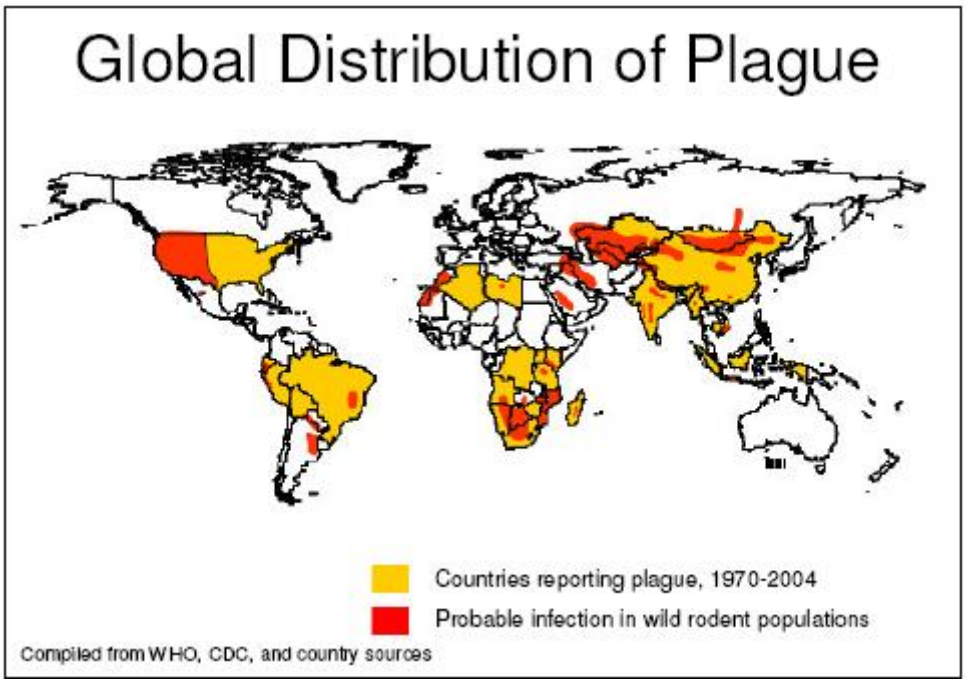

Figure 1. Global Distribution of Plague [cited 2008 Jul] Available from: < http://chppm-www.apgea.army.mil/documents/FACT/PlagueJusttheFactsOctober2007.pdf>

2.1.4.1 Significance of Plague Distribution in FSU/CIS

Forty-three natural foci are found in the southern and south-eastern regions of the former Soviet Union, which are essentially different in their epizootic as well as epidemic activities. The natural-foci were strategically located in 11 republics of the former Soviet Union consisted of six Institutes, 29 regional Anti-Plague stations, (APS), 53 field APSs, and about 200 specialized teams in epidemiology. It covers more than 216 million hectares or 9.6 % of the territories of the FSU. Analysis of available sources from archives and literatures documented from the year 1875 to 1997 indicates that there were an incidence of 10,453 human cases of plague in the FSU, whereby 9,054 (86.6 %) of them died (NARKEVICH et al. 1991, KOKUSHKIN. 1997, ANISIMOV et al 2004). In a continental-geographical demarcation, the majority of cases (5,574) were registered in the European part and less cases (4,879) in Asian part. Based on the epidemiological surveillance 1,224 (11.6 %) cases were registered from non-enzootic territories and 9,195 (88.4 %) cases were registered from natural foci territories. Investigation done on sources of infection, indicated that contacts with contaminated excretes of animals, or contaminated animal products like camel meat, as the main sources of infection. Only 34 cases (0.4 %) were reported as laboratory-acquired, direct contact in working with the pathogen. Investigations made on location of residence showed that, about 85 %, 14% of the cases were recorded from people living in rural area, and urban residents respectively (NARKEVICH et al. 1991, KOKUSKIN. 1997).

The 43 natural foci were classified in 5 regional divisions, the Caucasian region which includes 12 natural foci, (fig. 2), designated with number [1, 4-13, and 39], North Pre-Caspian region includes

number [2, 3, 14-17, and 43], Central Asian Desert region consist of number [18-30 and 42], Central Asian Mountain region consist of [31-35 and 40] and the Siberian region that covers natural foci [36-38 and 41]. Studies done on the nature of out-breaks indicated that most of the epizootic activities of the natural foci were/are recurring. It was also recorded that constant epizootic activities were found in Caucasian region [1, 4, and 5] (NIKOLAEV. 1972, BALAKHOHOV. 1991, KOKUSHKIN. 1997, ANISIMOV et al. 2004). For several decades, hundreds of plague experts in the FSU and the neighbouring countries like Mongolia and China were involved in field research studies of natural foci. Millions of rodents and fleas habiting in all types of natural foci were investigated. From 1920-1989 alone, bacteriological investigation was done on about 30 millions of rodents and about 102,095 strains of *Y. pestis* were isolated. Serological investigation revealed, a detection of specific antibodies from 106,349 animals (NARKEVICH et al.1991).

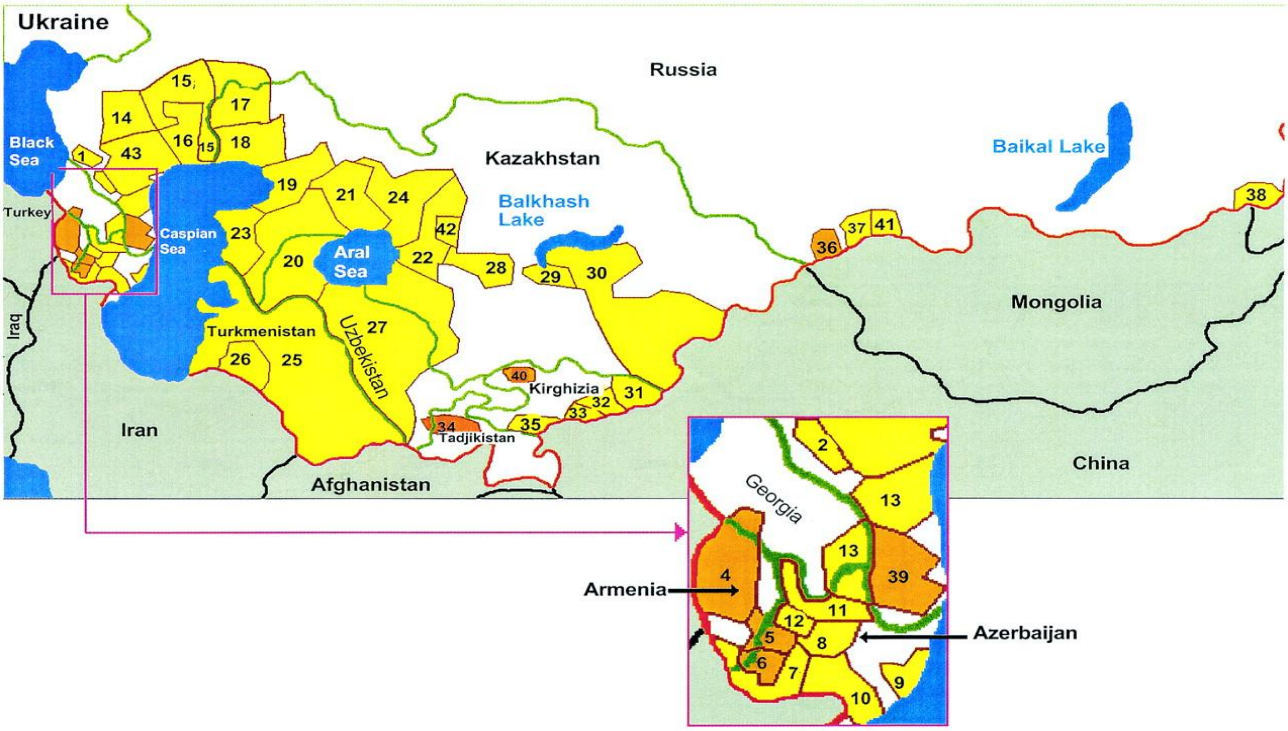

Figure 2. Distribution of natural plague foci in the FSU.

The red line indicate the FSU frontier; green lines indicate frontiers of states of FSU; brown lines indicates boundaries of the plague foci; black line indicates frontiers of other countries. Plague foci are yellow (for foci containing the main[1] *Y. pestis* subspecies) or, light brown (for foci containing non main[2] *Y. pestis* subspecies). (Figure adapted and compiled with permission from Anisimov et al., 2004 refered to (Balkhonov, 2000, Kokuskin, 1995, Nikolaev, 1972, Sludiski, 1998).

1. According to FSU/CIS classification the main subspecies are called *Y. pestis pestis*

2. To non main subspecies belongs *Y.pestis altaica; Y. pestis Caucasian; Y. pestis hissarica, Y. pestis ulegeica (*APARIN and GOLUBINISKI, 1989*)*

In order to specify strains isolated from natural foci, studies done on 257 samples of *Y. pestis* strains isolated from 31 natural foci in the FSU and neighbouring countries revealed that 10 % the strains obtained additional cryptic plasmids. For instance, strains isolated from Volga-Ural sandy focus, showed that 4 % of the isolates lacks *pPst*; 1.3 % of the isolates lacks *pCad*; 1 % lacks both *pCad* and *pFra*; 0.7 % lacks *pPst* and *pCad*; 0.3 % lacks *pFra* from a total of 18 % of the cases studied. Additional cryptic plasmids with a molecular weight of 30 MDa, 70 MDa were found in 5.7 %, 1.4 % of the isolates, respectively (DIMITRYUK et al. 1997, STEPANOV et al. 1975, 1981). Similar results were obtained from the Talas, Central-Caucasian, and pre-Balkhash foci, where strains isolated from these regions harbored a 20 MDa cryptic plasmid. Suggesting, that plague microbes circulating in natural foci of this region are highly diverse in comparison to strains known in Western Hemisphere, strains that harbors all three classical plasmids, *pFra, pCad* and *pPst* (WAKE et al.1983, BRUBAKER. 1991, CHERPANOV. 1991, FILIPPOV. 1990). Another similar investigation of 1,020 *Y. pestis* strains isolated from 44 counties of Yunana in China and the border of China-Myanumar were found to carry plasmids of nine different molecular weights, that leads the experts in China to classify them in plasmidovars (DONG et al. 2001) rather than biovars which is conventionally used in the Western hemisphere. The fact that plague microbes circulating in natural foci are surviving according to the law of nature in ecosystem, "survival of the fittest," they are dynamic, diverse, resistance to environmental changes than strains stored in Laboratory, for research purposes. Thus, considerations must be taken in processes of developing an efficient, universally protective and safe vaccine. Vaccine developed based on single strains stored-freezed in laboratories for several decades, must be proofed in providing efficient protection against intraspecific diverse strains, which are known as virulent in experimental animal models. Current vaccine development against plague in Western hemisphere focus on two major immunogenic proteins F1 and V, and the success of new candidate vaccines are majored based on protection from challenges with limited strains, with less or no genetical and phenotypical variations and mostly the maternal strain from where the antigens are extracted.

2.1.5 Mode of Transmission and Description of Disease

Plague is primarily a zoonotic infection, a disease of rodents and their flies that can infect human commonly via bite of an infected flea. It is a very severe disease in people, with case fatality rates of 50-60 % if left untreated (DENNIS. 1994, WHO. 2000). The most common mode of transmission of *Y. pestis* to human is by bite of infectious fleas. Less frequently, infection is caused by, a) direct contact with infectious body fluids or tissue while handling infected animal, b) inhaling infectious respiratory droplets or other infectious materials, e.g. laboratory-generated aerosols containing *Y. pestis* (fig.3). The classification of inhalation as less frequent is overlooked. The fact that plague outbreak in India (DENNIS. 1994) Madagascar (RATSITORAHINA et al. 2000) and recently Democratic Republic of Congo (WHO. 2006) were the pneumonic form, were fatal and the bacilli were isolated from cough droplets, which indicates out-coughed bacilli are contagious. Thus,

plague is a true respiratory disease and this route of transmission makes it a potential agent of biological weapon interest (DOMARADISKIJ. 1998).

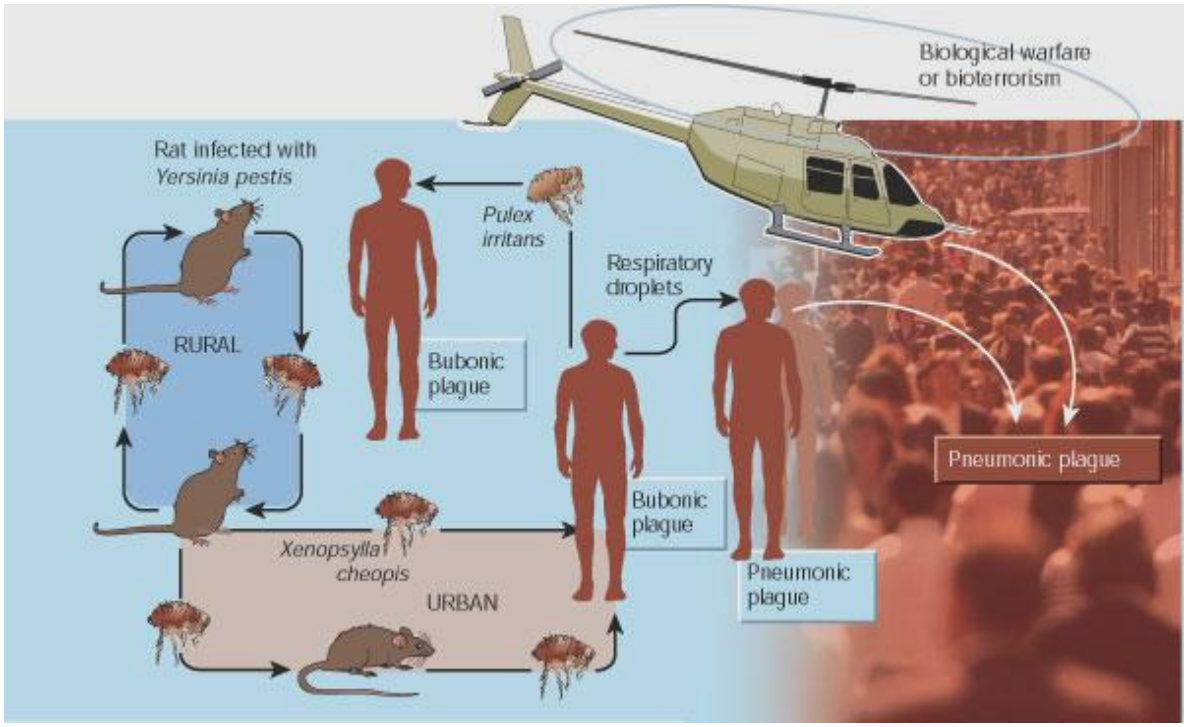

Figure 3. Y. pestis and its life cycle

How plague is transmitted. The causative organism, the bacterium *Yersinia pestis*, has a rodent reservoir. The rodents' fleas, such as the oriental rat flea, *Xenopsylla cheopis*, acquire *Y. pestis* from a meal of infected blood, and transmit the bacterium primarily to other rodents or to humans, causing bubonic plague in people. Human-to-human transmission can also take place, through the human flea *Pulex irritans*. Pneumonic plague is less frequent but even more severe; it is transmitted from person to person through respiratory droplets, or even by artificially generated aerosols, containing *Y. pestis*.[cited 2008 Jun 12]. 2001; Data adapted from Cole and Buchrieser. Available from:<http://www.nature.com/nature/journal /v413/n6855/fig_tab/ 413467a0_ F1 html>.

There are few reports concerning the possibilities of domestic animals in transmitting the infection. All most all of the literature concentrates on rodents. Nevertheless, in the south-western USA several human plague cases originated in cats and were transmitted via cat bite, scratch, or contact with infected body fluids, such as respiratory droplets (DOLL et al. 1994). Twenty-three cases of cat-associated human plague (5 of which where fatal) occurred in 8 western states from 1977 through 1998 which represents 7.7 % of the total 297 cases reported in that period (GAGE et al. 2000). Experimental cats fed on plague-infected rodents and subcutaneous inoculation demonstrated that all were ill (WATSON et al. 2001). 3 out 13 reported cases of human plague in USA were transmitted by domestic dogs (CDC. 2006). A sudden death of an extended family of 12 human deaths in March 1998 among the residents of a high-Andean community in Ecuador showed evidence of pneumonic plague. The family was believed to have an exposure to sick guinea pigs. Five of 14 dogs living in the community were tested seropositive for plague antibody providing evidence of a recent epizootic plague in the area (GABASTOU et al. 2000). Pneumonic plague outbreaks also played a great role in the Islands of Madagascar; family members of 18 individuals died within two weeks (RATSITORAHINA et al. 2000).

According to KUNIZA. (1997), analysis of various factors of 470 epidemiological outbreaks of plague , and single cases registered in the last 95 years in the territory of the former Soviet Union natural foci, showed that, next to the "portal type" of disease transmitted by rats, infected domestic camels known as "Utility Camel-type" and consumption of contaminated camel meat were responsible for about 81.5 % of the total outbreaks in summer-autumn season in this particularly investigated region. The role of domestic animals in transmition or as reservoir for plague microbe is not well investigated.

Plague exists in natural enzootic cycles involving wild rodents and their fleas in certain regions of Asia, Africa, the Americas, and extreme south-eastern Europe near the Caspian Sea. The natural foci may be in apparent, with no transmission to human or associated with sporadic transmission to humans. Epidemic of plague occasionally occurs when the disease spreads from rodents into the populations of rats that live in human habitation (ANISIMOV et al. 2004). Because of the high case-fatality rate and the epidemic potential of this disease, plague is designated a class I notifiable disease and thus is subject to International Health Regulations. These regulations require that all suspected cases must be reported to and investigated by public authorities and that confirmed cases are reported to the World Health Organization in Geneva, Switzerland. Maintenance of plague in nature is absolutely dependent upon cyclic transmission between fleas and mammals. It was known that, the oriental rat flea (*Xenopsylla cheopis*) alone, the classic vector for plague can ingest from 0.03 to 0.5 µl of blood what at least insures ingestion of 300 *Yersinia pestis* organism (HINNEBUSH and SCHAWA 1993). Based on animals data, at list 100 organisms are estimated to be infectious via the respiratory routes. Infection caused through aerosol, such as in case of bioterrorism, that causes a primary pneumonic plague, is the most feared and fatal form. In 1970 the World Health Organization (WHO) analysis suggested that "in the worst-case scenario" The release of 50 Kg of *Y. pestis* as an aerosol over a city with five million inhabitants could results in 150,000 cases of pneumonic plague, and the 360,000 of those affected would be expected to die.

The bacilli would remain viable as an aerosol for one hour for a distance of up to 10 Km. Inhabitants trying to flee would spread the disease further. These statements did not take into consideration of secondary cases that would occur subsequent person- to- person contacts and capability of causing pandemic.

Y. pestis infection in human mainly occurs in one of three primary clinical forms. It can appear in human in bubonic (80-90 %), septicemic (10-13 %), and pneumonic (1 %) forms of primary cases (fig. 4). Pneumonic plague is the most deadly form of the disease, with an incubation period of 2-4 days, fatal within 24 hrs if untreated (CHERNIN. 1989, WHO. 1992, 2000, CDC. 1992, DENNIS. 994). The classic disease in human bubonic plague, results from flea bite, or direct contamination of an open skin lesion by plague-infected material. Following inoculation, a local cutaneous proliferation, not usually clinically evident, ensues. In some cases, a vesicle, pustule, or ulcer develops at the inoculation site (POLLITZER. 1954, CAMPBELL and DENNIS 1998). The infection spreads via the lymphatics to the regional lymph nodes, causing inflammation and swelling in one or several nodes (buboes). Buboes may occur in any regional lymph node sites. After an incubation period of 2 to 6 days, patients typically experience a sudden onset of illness characterized by headache, shaking, chills, fever, malaise, and pain in the affected regional lymph nodes and may not be clinically enlarged at this stage. Progression of symptoms is usually rapid with regional lymphadenitis becoming excruciatingly tender and painful (WHO. 2000, CDC. 2005).When a superficial bubo is not found in a patient suspected to be infected with *Y. pestis*, the primary lymph node involvement may be present in deeper areas of the body including mediastinal and intra-abdominal lymph nodes. In this latter circumstance, abdominal pain, suggestive of appendicitis, colitis, enteritis or cholecystitis may represent the patient's principal complaint (BUTLER. 1983, HULL et al. 1986, 1987, VON REYN et al. 1977, WHO. 2000).

Primary septicemic plague is a progressive, overwhelming blood stream infection with *Y. pestis* in the apparent absence of a primary lymphadenopathy. The presence of rapidly replicating gram-negative bacilli in the bloodstream initiates self-perpetuating immunological cascades typically linked to host response to severe injury; in this case, the agent inciting injury is bacterial endotoxin (ALBIZO and SURGALLA 1970). The host response may result in a wide spectrum of pathological events including disseminated intravascular coagulopathy (DIC), multiple organ failure (MOF), and adult respiratory distress syndrome (ARDS) (BUTLER. 1974, CROOK and TEMPEST 1992, WENZEL et al. 1996). Disseminated intravascular coagulation can lead to arteriolar thrombosis, hemorrhage in the skin, serosal surfaces, and organ parenchyma, and sometimes results in acral cyanosis and tissue necrosis (DENNIS et al. 1997).

Primary pneumonic plague follows direct inhalation of aerosolised droplets containing *Y. pestis,* is thus, the infection of the lungs. It is the most fulminating and fatal form of plague. The incubation period is usually 1-3 days. Disease onset typically manifests by the sudden onset of chills, fever, headache, body pains, weakness, and chest discomfort. Cough, sputum production, increasing chest pain, difficulty in breathing, hypoxia and hemoptysis become prominent as the disease rapidly progress. Segmental pneumonia may progress to lobar pneumonia and then to bilateral lung

involvement and leads to respiratory distress syndrome. Pneumonic plague must be considered highly contagious whenever it occurs, person-to-person transmission, is reported in Madagascar, where seven male and one female died within 20 days. The source of infection resulted from their active participation in the funeral ceremonies and attendance on patients who died from pneumonic plague (HEATH. 1998, RATSITORAHINA et al. 2000, PERRY. 1997)

Plague pharyngitis results from contamination of the oropharynx with the *Y. pestis* infection material, is clinically similar to streptococcal or viral pharyngitis. It is likely that the diagnosis can mislead until there is laboratory identification of the *Y. pestis* in a throat culture (CROOK and TEMPEST, 1992). Meningeal plague usually occurs a week or more after the onset of bubonic or septicemic plague. It is often associated with delayed, inappropriate, or bacteriostatic antibiotic therapy and is more common in patients with axillary buboes (BECKER et al. 1987, CIDRAP 2003).

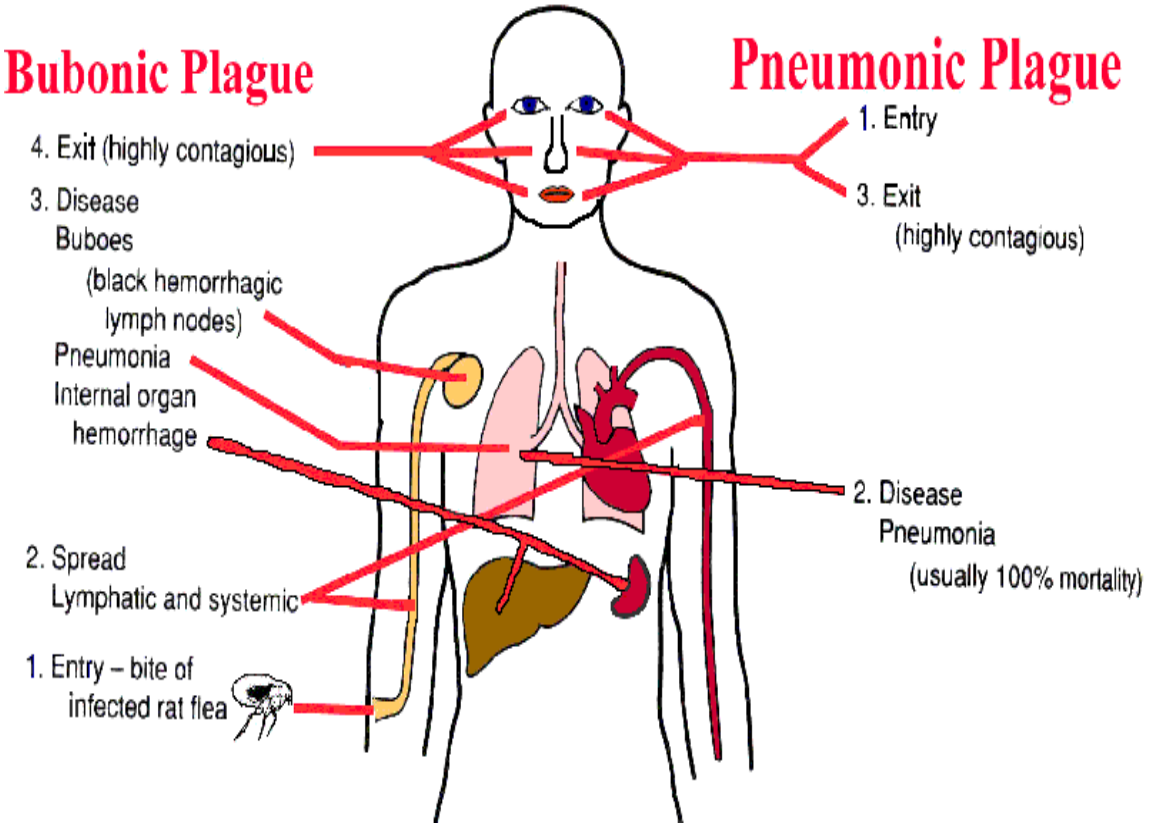

Figure 4. Plague clinical types: [cited 2007 May 23] Available from: <http://www.kcom.edu/ faculty/ chamberlain/website/lectures/lecture/plague.htm>

2.1.6 Potential as Biological Weapon

Biological warfare agents are defined as "living organisms, whatever their nature, or infected material derived from them, which are used for hostile purposes and intended to cause disease or death in man, animals and plants, and which depend for their efforts on the ability to multiply in the person, animal or plant attacked" (SPENCER and WILCOX 1993). Many such agents are zoonotic and have a considerable impact in Human and Animal health. The plague microbe, *Y. pestis* is one of it, that can cause disease both through endemic exposure and as biological warfare agent. Plague has a long history to be used as biological weapon, possibly the first known biological agent in human history. Plague is known for its notorious pandemic waves world wide throughout human history and transmitted by flea vectors such as deliberate spreading diseases through arthropods, the so called the vector effect of biological warfare (WHEELIS. 2002). Its modern application in warfare started in 1930s with Japan (INGLESBY et al. 2000). Germany and Soviet Union also conducted their investigation in this area around the same time (KIRBY. 2005, GEISELLER. 1998). During WWII, a secret branch of Japanese army, Unit 731, is reported to have dropped plague-infected fleas over populated areas of China and conducted researches on human in occupied Manchuria under the cover of research units named by General Ishrii Shiro (HARRIS. 1997). One of Ishii's greatest achievements was his use of the human flea *Pulex irritans*, as a stratagem to simultaneously protect the bacteria and target humans. This flea is resistant to air drag, naturally targets, and human flea can infect a local rat population to prolong an epidemic. Infected fleas may regurgitate up to 24,000 organisms in a single feeding (KIRBY. 2005). The experimental studies of this observation were done on prisoners as a final target to know, how effectively, can a human be infected with plague from fleas and pathological investigation, autopsies were conducted on some plague victims, even while they were still alive (GAGE and KOSOY 2005, KIRBY. 2005). The Japanese apparently used plague as a biological warfare agent in China several times during World War II. In any event, in the words of Pentagon analyst Andrew Weber quoted by ORENT. (2004), in comparing Japanese activities, it should be remembered that the Soviet or USA bioweapons researchers "never killed anybody". There are degrees in bioweapons work as in anything else: none of the Americans or Russians bioweapon scientists can be compared to the Japanese who worked on the infamous Units in Manchuria. They worked with human subjects on diseases that range from antharx to yellow fever. They researched human reactions to plague, typhoid fever, paratyphus A and B, smallpox, tularemia, glanders, tetanus, cholera, scarlet fever, tuberculosis, salmonella and other countless diseases that were endemic to the communities and surrounding region. No one has been able to catalogue completely all the maladies that the various death factories in Manchuria did on human guinea pigs. Testing pathogens on humans was only a single chapter, albeit a major one, in Ishii's BW enterprise, other secret research carried out by him and his associates had the two fold purposes of determining: 1) methods of culture of biological warfare agents; 2) methods of dissemination of plague, anthrax and glanders. They were also engaged with pathogens that kill plants and animals (CAUDLE III. 2007, BELLAMY and FREEDMAN 2001) and the actual number of people who died in Ishii's

bioweapons attacks and experiments from the year 1931-1945, in different locations will never be known, but the toll of the dead may have reached millions interviews and written reports found in boxes after Japan surrender contained autopsy reports that cover glanders, plague and anthrax in length from 350 pages to more than 800 pages (HARRIS. 1997, GEISSELER. 1998).

The Canadian's worked in ways different from others, developing biological weapons at Canada'sf Grosse Ile- a secret germ warfare research facility-labored to produce Anthrax for allies during the WWII. The intent was to create a colony of fleas for use in combination of with both plague and murine typhus. This concept of using a single vector to spread two different diseases simultaneously was an innovative approach, and was titled as "Entomological warfare" (USAMIID'S. 2004, KIRBY. 2005).

2.1.6.1 The Soviet Biological Warfare and the Anti-Plague System

The USSR possessed a unique national public health system that included an agency named "Anti-Plague Institute." Its mission was to protect the country from highly dangerous diseases of either natural or laboratory acquired infections. The predecessors of the Soviet API, called Special Commission for the Prevention and fighting against Plague entitled (KOMCHUMA), as well as research institutes specializing in the studies of plague and cholera that occurred in various regions of the empire, experimental and field laboratories engaged with epidemiological surveillance, security, quarantine activities were established during the Tsarist period of Russian empire (ONISCHENKO et al. 1999).

The Soviet API has also passed through two eras: the "classical" era (1928-1972) and the "modern" era (1973-1991). The USSR, like other nations supported offensive BW programs during this time, used the "classical" microbiological techniques of mutation, selection, and propagation to weaponize pathogens such as*, Y. pestis, B. mallei, B. anthracis, F. tularensis Rickettisia prowazekii, as* well as viral diseases such as *Crimean-Congo haemorrhagic fever* by UV light, X-rays or chemical agents most efficiently after WWII (OUAGRHAM-GORMLEY 2006, OREINT. 2004, NARKEVICH. 1991). The main task of the APS in this period was mainly to discover plague natural foci, identifying the main carierries classified as primary, secondary and random carriers and developments of methodology of epizootics, new treatment and developments of vaccines against plague, typhus, cholera, brucellosis, anthrax and tularaemia (WESTERDAHL and NORLANDER 2006, DOMARADSKIJ and ORENT 2006, FRISCHKNECHT. 2003).

The FSU BW facilities consisted of, a system under military control and the second top-secret programmes under civilian cover, called "The Biopreparat complex", a new network of BWP, began in the early 1970 (CNS. 2002). Another group of facilities worked on microbial agents harmful to livestock and plants, one of such facilities was, the Scientific Research of Agricultural Institute in Kazakhstan (NISKHI) established in 1958. These facilities were administered by the 15th Directorate for Biological Protection of the Soviet Ministry of Defense; Fvozrozhdeniye Island in

the Aral Sea was the main testing ground for biological agents developed at Ministry of Defense (RIMMINGTON. 1996). Leading Bioprepart facilities included the State Scientific Center of Applied Microbiology in Obolensk, the Institute of Immunological Studies in Lyubuchany, the State Scientific Center of Virology and Biotechnology (known as Vector), to note some of the prominent establishments. In addition to the aforementioned centers within the offensive part of the Soviet BW program, other facilities were involved mainly in prophylactic and vaccine developments (CNS. 2002).

The system of Anti-plague research institutes and field monitoring stations under the authority of the Main Directorate of Quarantine Infections of the USSR Ministry of Health include the Microbe Scientific Research Anti-Plague Institute in Saratov, the Rostov Anti-Plague Institute, The Volgograd Scientific Research Anti-Plague Institute and the Irkutsk Anti-Plague Institute for Siberia and the Far East (OUAGRHAM-GORMELY. 2006). The establishments were located in different regions of the territory and exchanges of top-ranking specialists between the Anti-plague institutes and offensive BW facilities, their participation in some offensive BW program cannot be ruled out (ZILINKAS. 2006, ALIBEK. 1999).

Some of the worlds leading experts on pathogens of BW importance in FSU like Igor V. Domaradiskij, K. Alibek, L. Melinkov, Sergei Popov, and other prominent scientists of the Soviet bioprogram studied plague, anthrax, tularemia, brucellosis, typhus, Q-fever, smallpox, botulinum toxin, Venezuelan Equine Encephalitis, and other viral infections their causative agents were designated as “the dead list pathogens” in Obolesk, Vector, Saratov, and other similar research institutions, located in the territory of FSU (DOMARADSKIJ. 1998, OUAGRHAM-GROMLEY et al. 2006, ALIBEK. 1999, 2003). The experiments were conducted on horses, monkeys, sheep, and donkeys, and on small laboratory animals such white mice, guinea pigs, and hamsters. Lev Melinkov, one of the plague expert, in the FSU, who worked at Saratov Microbe Institute quoted by ORENTS (2004) said, “The Soviets are born to research on plague”. Indicating a large number of highly competent expertise commitment and main concern in plague research at the state level. It is also known that, the territory of FSU includes one of the world’s most virulent reservoirs of the plague pathogen “hot-spot”, with high intraspecific diversity in quantity and quality in the world documented through extensive years of research, monitoring and control of plague disease outbreak by API experts including the neighbouring countries like Mongolia (ANISIMOV et al. 2004, ONISCHENKO et al. 2004).

In the FSU there were two faces of plague work, the plague fighters and the plague engineers, those who worked to make plague a weapon and those who combat the endemics in the region. The main achievements of the experts in offensive area were, genetical modification of the pathogens, so that they will be resistant to vaccine and/or antibiotic treatment and development of vaccines. Sometimes these two faces belonged to the some person. Igor Valerianovitch Domoradskij, specialized in microbiology, had been deputy director of the Interagency Science and Technology Council on Molecular Biology and Genetics, the “brain center” as Domaradiskij puts it–of the entire Soviets’ program. The council designed the overall aims and methods of the Soviet bioweapon

system. He discovered the methods of detecting plague through plague phages that promotes him to be a deputy director (ORENT. 2004, DOMARADSKIJ and ORENT 2006).

In the mid 1960th the Anti-plague Institute at Rostov, where Domaradiskij made his investigations was shifted its emphasis from basic research to biodefense under orders from Moscow, the Soviet called it problem №.5. He also developed a "dry" plague vaccine using a live strain of *Y. pestis*. This vaccine has an additional property of being able to withstand antibiotics, so that in an emergency it could be given concurrently with antibiotics. His team also discovered that in the plague microbe a foreign plasmid can be introduced, whereby the bacteria requires a new properties including antibiotic-resistance (CBW. 1998). In the year 1979, Domaradskij's major scientific discovery was that *Y. pestis* not only could accept foreign plasmids, but had three native plasmids of its own. This novel discovery was kept as the secret of secrets, which was never published outside the closed world of Biopreparat system. The discovery of plasmids was to revolutionize the study of the plague, mainly for military purpose. Plasmid as it is known as a working horse molecular biotechnology; this discovery was a triumph of high degree for the plague experts (ORENT. 2004, DOMARADSKIJ and ORENT 2006). Three years later, FERBER and BRUBAKER (1981), the distinguished American plague researcher first published their discovery of plasmids.

The "modern" era of the Soviet BW activities began right after the discovery of DNA technolology, which stimulated the Soviet government to increase its own efforts in the field of biotechnology including applications of military purposes (ALIBEK and HANDELMAN 1999, ORENT. 2004). Two major offensive institutions codenamed "Ferment" and "Ekology" were established. Ferment was directed at solving problems related to weaponizing pathogens for the use against human, while Ekology was programmed to develop against animals and plants (ZILINISKAS. 2006). Gene engineered strains of *B. anthracis* resistant to tetracyclines and penicillins were also reported in Russia (STEPANOV et al. 1996, ALIBEK. 1999).

On 10 April 1972, the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction was opened for signature. It entered into force on 26 March 1975, after the deposit of the instruments of ratification by 22 signatory governments, including the governments of the Soviet Union, the United Kingdom and the United States as well as seventy-seven countries, which bans the development, production, use and stockpiling of BW agents. But research and development continued in FSU (MDN. 2004). Three years later Domaradskij and Zhadanov (the famous smallpox eradicator) developed a plan levelled the Five Principal Directions, which established the future course of biological weapons research. Genetic modifications of existing strains were at the heart of this program: viruses and bacteria modified for great virulence, stability, durability in external environment, and genetic resistance to vaccines and antibiotics (ORENT. 2004, ONISHENKO et al. 1999). In particular, they envisioned adding short chains of proteins, or peptides, to bacteria and viruses, which create diseases with entirely new and different symptoms, and which would make the infections more difficult to diagnose and treat (WESTERDAHL and NORLANDER 2006). They planned to divide this work up among many different institutions, some of which would

concentrate on basic research, and others which may assign to work directly on highly dangerous infections. Head of these laboratories were “Vector” and “Obolensk” (CNS. 2002; ORENT. 2004)

Vector Laboratories, built by convict labor in 1974, is a gigantic complex, dedicated to research on viruses, it was the USSR’s principal fiefdom for bio-weapons research on viruses until 1992. Obolensk was the chief bacteriological weapon laboratory, which had a close connection with Kirov military laboratory, also specialized in highly contagious bacteria (CBW. 1998, WARRICK. 2002).The Soviet cover was partially blown in 1979 when a massive outbreak of anthrax affected a large area around the Ural city of Sverdlovsk where at least 96 people were infected and 66 people died. The true figures, no doubt, are higher (MESELSON et al. 1994, CBC. 2004, WILKENING. 2006).

By the late 1970s, the system was composed of 87 facilities engaged in disease surveillance, research, production and testing of vaccines and laboratory equipment, and training of civilian and military personnel. The system employed a staff of 14,000, including 7,000 scientists whose expertise broadened beyond plague to other endemic zoonotic diseases, such as anthrax, brucellosis, tularemia, and Congo-Crimean hemorrhagic fever. Most importantly, the Anti-plague system stretched beyond Russian borders into Central Asia, the Caucasus, Ukraine, and Moldova, with facilities strategically located in 11 republics (OUAGRHAM-GROMELY et al. 2006).

Plague has always been the favourite bacterial weapon of the Soviet military - as Domaradskij puts it, after an initial bioweapon attack, “plague spreads from man to man, and further effort on the part of military is not necessary”. He found himself in endless, relentless conflict with Urakov. In 1987, Domaradskij left Obolensk, where he spent twenty-three years in the anti-plague system of the Soviet Union (DAMARADISKIJ and OREINT 2006). A year before Domaradiskij left Obolensk Sergi Papov, who now works in Virginia, USA, was brought in from Vector at Novosibirisk to do genetic engineering experiments on plague and other pathogens. As papov puts it quoted by Orent “plague was already a king in Obolensk. Hundreds of scientists studied plague in several of the institute’s departments. The microbe was encoded as Agent №.1 on the secret list of bacteria used to make biological weapons”. Papov and his teams, biochemists and virologists, were engaged in synthesizing the DNA to produce peptides from various human immuno-chemicals for eight years and inserted them in to mouse poxvirus and vaccina virus, stands-ins for smallpox virus, capable of regulating production of other proteins in artificial viruses. These were feats of almost unimaginable complexity; such experiments showed that, at least in theory, viruses could be genetically altered to express new, unexpected, and deadly properties. He also inserted synthesized DNA sequences into plasmids that could be delivered directly into the cytoplasm of the microbe’s cells, which were designed to yield a variety of effects, including terrifying new forms of plague, anthrax, glanders, legionella, and tularemia (OUAGRHAM-GROMMY. 2006, ALIBEK. 1999, CBC. 2004, POPOV et al. 1997)

A patient infected with these genetically altered plague strains would first contract what appeared to be a typical pneumonic plague infection, immediate treatment with appropriate antibiotics, which would cause the plague bacterial cell to break apart. The patient might appear to recover,

but within the body, the dying bacteria release their cargo of artificial peptides, which in turn would cause paralysis, tachycardia and finally dies from heart stroke or fatal paralysis, which the scientists called “Recombinant Plague” (WESTRDAHL and NORLANDER 2006, DOMARADISKJI and ORENT 2006). Another scientist, Sergei Netesov, Department director of Vector laboratories, was known as the originator of the whole concepts of chimeras: genetically engineered viruses made of two component parts – for instance smallpox and Venezuelan equine encephalomyelitis (VEE), smallpox and Ebola, that had promoted him to depute director of Vector in Novosibirsk. The name of the program he directed was known as *ОХОТНИК* (okhotnik-hunter). He proposed Plague–VEE chimera, the object being, a victim of this chimera would be treated for plague with appropriate antibiotics, which would kill the plague microbe. But shattering the bacteria cell walls would release VEE directly into the lymph node or bloodstream; then straight to the brain, the victim dies of encephalitis and the “Okhotnik project was successful” Netesov and Popov quoted by ORENT (2004). Papov remained in Obolensk until 1992. Another study was very surprising and far more ominous. When they tested their Plague-diphteria chimera on monkeys, they found that it could overcome immunity to live plague vaccine. In other words, the new plague chimera was, at least to some extent, not only lethal at the lower doses than normal weaponized plague but also vaccine-resistant. A hyper-lethal, vaccine-resistant, antibiotic-resistant plague weapon would be the most powerful biological weapon of all- a veritable Andero-meda Strain, a Black Death for the twenty-first century. There is no reason to think such a weapon has ever been made. However, the technology may now exist to produce it.

In the United States, the main biological weapons threats are considered smallpox and anthrax. Nevertheless, the Russians believed that plague is the most dangerous bacterial threat agent. As anthrax and plague genomics expert Paul Keim of the Northern Arizona University puts it, the Russian and U.S. biodefense programs are like mirror images of each other: we fear anthrax, since both lethal and extremely durable in the environment, and they fear plague, for its virulence and transmissibility (DOMARADIKJI and ORENT 2004).

Since the mysterious anthrax attacks in late September 2001 in the United States, it was proofed anthrax contamination can spread throughout the building, and how difficult it is to decontaminate the area to remove the spores. But anthrax does not spread from person-to-person, probably because anthrax is not a true pulmonary infection. It can seed in itself in the lungs, but soon it moves to the lymph nodes and the chest. You can’t cough it out in a contagious form: coughing produces big droplets, which can’t be inhaled, and the bacteria in any event are in the vegetative-growing-state and are not infectious. Only spores are infectious. Plague is a very different disease from anthrax: the chief danger from plague, aside from lethality, is its ability to spread. Unlike anthrax, it grows in the lungs; it produces infected sputum; it is coughed out and transmitted from person-to-person, lung to lung. Coated in sputum in some manners, as bacteria naturally are, when they are coughed out, it becomes more stable in the external environment (not stable as anthrax), but stable enough, it can last for weeks on many surfaces under certain conditions and remain infectious. Plague in a frozen corpse can remain alive almost indefinitely.

The majority of bioweapon publications are concentrated on humans as a target “Anti-livestock” or “Anti-agriculture” weapons are less studied and published. But they can wreak economic havoc and even undermine a nation’s ability to feed itself. Under the Soviets, as many as six agricultural research centers and up to 10,000 scientists and technicians were believed to have been devoted to developing them, working under a shroud of secrecy that persists today and complicates efforts to keep dangerous materials. This facility is believed to posses more than a dozen viruses, including, foot-and mouth-disease (FMD), Newcastle, targeted to livestock and poultry production respectively (ONISCHENKO. 2003, ISTC. 2008).

Quiet a few of the best specialists of bioweapon program of the FSU made their way to the west and shared their awareness, talked and wrote their memories about the danger of the activities in which they were engaged for several decades, with deadly pathogens. To note some of them like, Popov defected to UK, Alibek to USA, who was the former first Deputy Director of Biopreparat from 1988 to 1992, he was famous for his methods of weaponizing anthrax and in his interview said “The Soviet Union has two main directorates responsible for developing and manufacturing biological weapons were stored at the Minster of defense facilities, for example [the] Kirov facility was responsible for storing plague, about 20 tons of plague” (ALIBEK.1999, BARRY. 1993).
In Todays Russia, the system consists of five research institutes (in Volgograd, Saratov, Stavropol; Rostov-on-Don and Irkustisk), 11 stations and 14 units in operation and in natural disease foci (ONISCHENKO. 2003). A unique system to control deadly endemic diseases and to prevent the importation of exotic pathogens from other countries that could threaten human populations, livestock, and crops under the institution designated “Anti-Plague System.” APS is the only one in its kind that operated for more than a century, is one of the achievements of the FSU (DOMARADISKIJ and ORENT 2006, ZILINSKAS et al. 2006) where the word “plague” was used broadly to refer to infectious diseases causing high morbidity and mortality. The threat of BW has increased in the last two decades, with a number of countries working on the offensive use of these agents. There are more than 10 countries around the world suspected to have offensive biological weapon programs (USAMRIID’S. 2004).

2.2 Burkholderia mallei

B. mallei, is a gram negative bacteria, that causes glanders, one of the oldest documented infection among solipeds recognized since Ancient Greece (LOEFFLER. 1886, BERSTEIN et al. 1909) Currently about 41 species of *Burkholderia* have been identified based on DNA-DNA homology, base sequence of the 16S rRNA, and various phenotypic characteristics (YABUUCHI et al. 1992, DSMZ. 2008). Although most species of *Burkholderia* are saprophytes or plant pathogen, two species, namely *B. pseudomallei* and *B. mallei*, cause serious and fatal diseases (DANCE. 1998), both are, nonsporing, bacillus with a "safety-pin" appearance using Methylene Blue or Wright's stain on microscopic examination (ASHDOWN. 1979, HPA. 2003, BOSSI et al. 2004). *B. mallei* is obligate pathogen, strict aerobe, catalase-negative, usually oxydase-positive, non-motile. Primary culture isolates require 48 hrs at 37.5 °C under aerobic conditions. In contrast, recent comparative genomic sequencing of *B. mallei* C-5 bacterial culture collection of SRCAM (Russian), originally isolated from horse corpse in Mongolia in 1967, and *B. pseudomallei*–141 isolated from patient in Vietnam in 1948 showed a great genome-wide intra- and interspecies variability. At the some time this investigation showed a high heterogenity in *B*. *pseudomallei* species, a low but detectable variability in *B. mallei* species (FUSHAN et al. 2005). The revealed differences included some putative virulence factors, new insertion sequences and a gene cluster of *B. mallei* that encodes proteins involved in the biosynthesis, export and translocation of capsular polysaccharide. The capsule has been described as a major virulence factor required for the genesis of glanders in animal model (DESHAZER et al. 2001).

The genome of *B. mallei* consists of two circular chromosomes and is found to be smaller than that of *B. pseudomallei,* 5.8 Mb and 7.2 Mb, respectively (NIERMAN. 2004). In similar fashion, *B. pseudomallei* has been characterized to be a strong secretor of proteases, lipases and phosoplipalse C via the secretory pathway (DESHAZER. 1999) although there role is not well known, whereas *B. mallei* was recently reported poorly secretes these protein, if at all (NIERMAN et al. 2004).

Most naturally occurring human cases of glanders result from close contact with infected animals or the agent itself for instance in laboratory. It may be more communicable than melioidosis. The infection dose of both organisms for humans is not known. One reported human case of glanders infection described on 5th of May, 2000, was a microbiologist working with pathogen at the USAMRID, Baltimore, USA, treatment with ceftiaxone and cephaexin did not improved the conditions of the patient. That indicates that *B. mallei* is capable to resist a number of antibiotics and able to relapse despite completing the therapy (CDC. 2000, SRINIVASAN et al. 2001).

For both pathogenic Burkholderia organisms, it is likely that the natural mode of transmission is mainly by inoculation into small abrasions. Infection by inhalation also occurs, and is the most likely route by which infection will occur following deliberate release, i.e., bioterrorism. There is circumstantial evidence that glanders may be acquired by ingestion. This is less convincing for melioidosis, although two recent outbreaks in Australia were linked to contaminated potable water supplies (INGLIS et al. 2001).

Glanders is one of the oldest documented disease of solipeds caused by *B. mallei* (LOEFFLER. 1886), but incidental infection also occurring in humans, carnivores and wild animals (MCGILVRAY. 1944; ALIBASOGLU et al. 1986). It is still endemic in parts of Africa, the Middle East, South and East Asia, Central America, and parts of some of Countries of FSU like in Kazakistan (MOTA et al. 2000, WAAG and DESHAZER 2005).

It can cause a subcutaneous infection known as farcy or can disseminate to cause the condition known as glanders. In humans, four forms of glanders are seen. The local cutaneous form, if the skin was the portal of entry. Pulmonary form, if the organism was inhaled, septicemic form if the organism is in circulatory system and the chronic form. The acute infection is characterized by necrosis of the trachobronchial tree, pustular skin lesion, and either a febrile pneumonia, or signs of sepsis, and multiple abscesses (DIADISCHEV et al. 1997, CDC. 2000; 1992; CFSPH. 2007). The generalized symptoms of glanders in humans include fever, muscle aches, chest pains, muscle tightness, headache, night sweat, weight loss e.t.c. (SRINIVASAN. 2001).

There have been too few human cases of glanders in the post-antibiotic era to make an accurate estimate of case-lethality. Some estimate that glanders is in over 50 % of the cases fatal despite antibiotic treatment and other say it may be similar to that of melioidosis, which is indeed 50 % of the cases, as well. Without treatment there is a 95 %-100 % fatality rate with all forms of glanders (CFSPH. 2007) and can be reduced to 20 %, if appropriate diagnosis is confirmed and treated very early (CRAVITZ. 1950, NEUBAUER et al. 1997).

There are several reports that describe in vitro susceptibility of *B. mallei* to antibiotics (HEINE et al. 2001, KENNY et al. 1999, BATMANOV. 1991, RUSSELL et al. 2000). These studies generally demonstrate most *B. mallei* strains exhibit resistance to first class antibiotics. Experimental treatment of golden hamster with seven highly effective chemotherapeutic agents against *B. mallei* showed no protection, none of the experimental animals survived (BATMANOV. 2001). Similar results were seen, in treatment of golden hamster with four highly effective antibiotics against a high dose of aerosol infection with *B. mallei* that did not protect the animals (ILYUKHIN et al. 1994). Although, there is little evidence on antibiotic treatments of glanders diseases in Human, some authors propose, that *B. mallei* might have the same characteristic of multi drug-resistant (MDR) as its' very closely related species of *B. pseudomallei*. *B. pesudomallei* is well known for its MDR and relapses after appropriately successful primary treatment of melioidosis patients and MDR in vitro studies are also not uncommon (BATAMANOV. 1997, DANCE et al. 1989a, LEELARASAMEE and BOVORNKITTI 1989).

2.2.1 Infection, Epidemiology and Transmission

B. mallei is a host-adapted pathogen that does not persist in nature outside its equine host (HOWE. 1950). It is primarily noted for producing diseases in horses, mules, and donkeys. In the past man has seldom been infected, despite frequent and often close contact with infected animals? This may be the result of exposure to low concentration of organisms from infected sites in ill animals and because strains virulent to equids are often less virulent for man. The clinical courses of glanders include fever, rigors, nasal discharges, fatigue, weight loss, decreased appetite, dyspnoea (SRINIVASAN et al. 2001, LOPEZ et al. 2003).
Human cases have occurred primarily in veterinarians, horse and donkey caretakers, and abattoir workers. It is also reported as a laboratory-acquired infection in human (CDC. 2000, CHRISTOS and ELLIOT 2001). Glanders also has been reported in carnivores that have fed on infected horse carcasses. Infection of four lions at Safari park in Napoly, Italy (BATTELLI et al. 1973, GALATI. 1973) was attributed to ingestion of contaminated meat from imported horses. ALIBASOGLU et al. (1986) assumed also that feeding infected raw horsemeat was the cause of an outbreak of glanders in lions at Istanbul Zoo. Glanders was an important cause of death among horses at the turn of 20th century. The elimination of equine glanders from Western Europe and North America was achieved by rigorous slaughter policy and aggressive control measures (FAO. 1997). However, it is assumed that it is endemic in many developing countries (KNIGHT. 1972). Glanders is currently limited to parts of Asia, Africa, the Middle East, and Asia (specifically Turkey, Syria, Iraq, Iran, Pakistan, India, Burma, Indonesia, the Philippines, China, and Mongolia) and possibly the Balkan states, former Soviet republics, Mexico, and South America (ARUN et al. 1999, RAY. 1984, STELLE. 1979, VAID et al. 1981, VERMA. 1981). Cross-reactions with serological tests for *B. pseudomallei* may confound estimates of worldwide distribution.

Human glanders can be acute, leading to a rapid decline in respiratory function, and if untreated, is nearly fatal. It is characterized by necrosis of the tracheobronchial tree, pustular skin lesions either a febrile pneumonia, if the organism was inhaled, or signs of sepsis and multiple abscesses if the skin was the portal of entry (SANFORD. 1991, JENNINGS. 1963). The preclinical stage of the human form lasts from 2 to 8 weeks and shows only constitutional symptoms. If it is not properly diagnosed in this stage it might mimic tuberculosis or syphilis (nonclinical glanders or “farcy”) that can afflict patients throughout their lives (MCGILVARY. 1944, CDC. 2000). Despite the efficiency of spread in the laboratory setting, there is no epidemic of human glanders reported. It can spread to man by invading the nasal, oral, and conjunctival mucous membranes, by inhalation into the lungs, and by invading abraded or lacerated skin. Aerosols from cultures have been observed to be highly infectious to laboratory workers (LEVER et al. 2003). Aerosol spread is efficient and there is no available vaccine or reliable therapy.

Intracellular confinement and persistence of *B. mallei* or *B. pseudomallei* cells, and their high virulence impede treatment of glanders and melioidosis (JONES. 1996, SHARMA. 2001). A comparative assessment of anti-bacterial preparations against experimental melioidosis, found that growing recurrence of melioidosis is connected with persistence of *B. pseudomallei* in the bodies of laboratory animals as L-forms, which retain their pathogenic properties and may reverse into the initial bacterial strain. It was proven that in case of melioidosis, carriage of L-forms in macroorganisms is accompanied with the development of delayed types of hypersensitivity (DTH) (BATMANOV et al. 2001), that might be also the case in *B. mallei* as well. Recently the presence of TTS (type three secretion) genes in pathogenic *Burkholderia* species is suggested to have a link to virulence are important to elucidate the contributions of TTS to virulence in these organisms (WINSTANLEY. 2000).

2.2.2 Multidrug Resistance

At present information on the effective treatment of human infection due to *Burkholderia mallei* with antibiotic is sparse. It is believed to have similar characteristics to the very closely related *B. pseudomallei*. Humans are accidental hosts of *B. mallei.* The majority of natural cases have been the result of occupational contact with infected animals (stablemen, veterinarians and slaughterhouse employees) (STEELE. 1979). One recently reported case of a human infection; a patient who worked with the agent was treated with cetriaxone (im). Ten days course of cephalexin did not improve the situation of the patient. Then after isolating the agent intravenous application of imipenen and doxcycline improved his symptoms rapidly (CDC. 2000, SRINIVASAN et al. 2001). *B. mallei* is regarded as the most dangerous microorganism to handle in the laboratory. Experimental treatment of golden hamster with 7 highly effective chemotherapeutic agents, like monocyclin, doxycyclin, ciprofolxacin, ofloxacin, biceptol, sulfatan, and rimapcin showed no protection, when the animals were infected s.c. with 100 LD_{50} or aerosol with 160 LD_{50} with *B. mallei C-5* virulent strain. None of the experimental animals survived (BATMANOV, 1997). *Burkholderia spp.* are able to survive within phagocytic cells, exhibits mechanisms of resistance against the immune system of the host (PRUKSACHA TVUTHI et al. 1990).

2.3 Burkholderia pseudomallei

The causative agent of melioidosis, *B. pseudomallei* was first recognized in 1912 by WHITEMORE and KRISHNASWAMI in Rangoogn as a glanders-like disease, motile organism due to its multitrichous polar flagellae in contrast to *B. mallei*, which is nonmotile organism that lacks flagella (MILLER et al. 1948, BOSSI et al. 2004), the reason why *B. mallei* is non-motile is not well studied, yet its genome contains numerous flagellar and chemotaxis genes, most of which appear to be intact, but recent comparative genomic analysis within three *Burkholderia* spp. made by NIERMAN et al. (2004), showed, that one methyl-accepting chemotaxis gene has a frameshift mutation. In addition, a 65-kb insert flanked by IS elements disrupts the *filp* gene, an essential gene for flagellum biogenesis (MALAKOOTI et al. 1994) and a frame shift mutation in the flagellum motor *motB* may the reason for amotility and missing flagellation. The DNA-DNA homology value between *B. pseudomallei* and *B. mallei* has been reported to be more than 90 %, and the sequences of the 16S rRNA of the two species matched completely (BAUERNFEIND et al. 1998).
B. pseudomallei is a facultative aerobic, opportunistic pathogen, catalase-negative and mostly oxidase-positive grows aerobically at an optimal temperature of 37 °C - 42 °C, Cultures have earthy/grape odour, which can be detected when the incubator is opened (HPA, 2003). In nature *B. pesudmallei* can survive with in a temperature ranging from 4 °C– 43 °C in water and soil for prolonged period without any form of nutrient and is tolerant to range of adverse environmental conditions such as low pH. The incubation period of the organism is unclear, but ranges from 2 days to 26 years are recorded (LEELARASAMEE and BOVORNKITTI 1989). The DNA of *B. pseudomallei* has a higher molar percentage of guanine and cytosine (G+C) residue of 68 % and spontaneous transformation has also been found in *B. pseuodomallei* (HOWE et al. 1971).

An Environmental isolates previously considered to *B. pseudomallei* have been shown to fall into two closely related groups on the basis of there abilities to assimilate arabinose and show differences in there DNA macrorestriction patterns and rRNA sequences (BRETT et al. 1997, 1998, SMITH, 1997, TRAKULSOMBOON. 1997). Those that can assimilate arabinose (*ara*$^{+}$*)* have been assigned a new species, *B. thailandensis*, and are considered as avirulent, whereas the isolates that cannot assimilate arabinose (*ara*$^{-}$*)* and are associated with melioidosis are retained within the species *B. pseudomallei* (BRETT et al. 1997, SMITH. 1997). Although, there are numerous correlations between virulence and disease presentation in melioidosis patients, it is not a consistent observation. Virulence did not correlate with the origin of isolation (i.e. clinical vs. environmental), since numerous *ara*$^{-}$ environmental isolates were highly virulent. That indicate virulence of selected *B. pseudomallei* isolates is variable, being dependent on environmental factors such as iron bioavailability, inoculum size and host risk factors in determining the clinical severity of melioidosis (ULETT et al. 2001). The genomic DNA from 22 isolates of *B. pseudomallei* obtained from clinical cases and soil habitats in Malaysia demonstrated high heterogeneity (HASSAN. 2001, BULANTSEV and LOZOVAIA 1985) and similar results were also observed in Thailand, in a comparative analysis of 371 clinical and 206 soil isolates (SERMSWAN et al. 2001).

B. pseudomallei, infects human, domestic and wild animals and is regarded as endemic to the southeast Asia and northern Australia, corresponding to the tropical latitudes between 20° N and 20° S (fig. 5) and currently melioidosis is categorized as an emerging disease. It has only been studied in small number of countries i.e., Australia, Thailand, Singapore and Malaysia; but it is assumed to be widespread in tropical South East Asia (DANCE. 1991, 2000, 2001, CHENG and CURRIE 2005). There is evidence that the disease might be endemic on the Indian subcontinent and in some states of Caribbean countries, and there have been reports from South Africa and the Middle East. There have been occasional reports of cases of melioidosis in temperate countries, including an outbreak in France in 1970 (MOLLARET. 1988) and travellers, military personells served in endemic regions and reported cases were imported from tropical areas (ALDHOUS. 2005).

There are reported instances of person-to-person spread of melioidosis, prenatal transmission from mother to child that caused abortion, cases of infection through sexual contact with patient among chronic prostatitis (ABBINK et al. 2001). Infection can be acquired in the laboratory (MCCORMICK et al. 1975) and Siblings with cystic fibrosis who immigrated from Australia to New Zealand were believed to be infected by person-to-person transmission (HOLLAND et al. 2001), nevertheless the infection is not highly communicable.

The clinical spectrums of melioidosis are protean, can manifest as disseminated or localized disease. In disseminated form, it is characterized by an acute and progressive course with septicemia and shock, with acute respiratory failure often develop into adult respiratory distress syndrome (ARDS) (MANEECHOTESUMWAN. 1999). In a localized form, it is usually associated with lung, liver, spleen lymph nodes, urinary tracts, or muscles (WHITE. 2001). Cases with localized cutaneous or subcutaneous abscesses or lymphadenitis can also manifest. Neurological melioidosis were also reported in humans and animals (LADDS. 1981, HPA. 2003, CURRIE. 2000). Bacteremic melioidosis with pneumonia carries has high mortality. Recent studies showed that, in case of pneumonia the mortality rate of melioidosis was 72 % compared to the non-pneumonia group (MUKHOPADHYAY et al. 2004). Another similar study reported in Australia showed a mortality rate with 67. 3 % (CURRIE et al. 2004). Both results demonstrate that melioidosis is a severe disease of public health and security concerns. The overall case-lethality rate of severe bacteremic melioidosis might approach 100 % if untreated, but can be reduced to approximately 40 % with optimal management. Localised melioidosis has a much lower mortality rate (4-5 %) (CURRIE et al. 2001). The microorganism can persist in the body for several years before emerging.

Cultural as well as clinical diagnosis of both pathogens is difficult. The infections are often misdiagnosed because they trigger multiple symptoms (NEUBAUER et al. 2005) that mimics those of other diseases (CDC. 2000). In parts of Asia where *B. pseudomallei* is endemic, this serial killer often commits its crimes without even being identified as a suspect, and portrayed as a “Silent killer” (CHENG AND CURRIE 2005).

Both *B. mallei* and *B. pseudomallei* are included in category B lists of 'critical biological agents' of the Centers for Disease Control and Prevention, USA (CDC. 2000). However, according to Russians ranking system both pathogens are included in group A, potential critical biological agents (KONDRIK et al. 2003). They have a number of features that makes them attractive candidates for deliberate release. The ability to cause severe, rapidly fatal invasive infections, the ability to initiate infections via aerosols, inoculation and possibly ingestion. Its' intrinsic resistance to many antibiotics, to infect a wide range of animals as well as humans, and long term persistence in the environment under suitable conditions (*B. pseudomallei*) (HPA. 2003, ROTZ et al. 2002) and currently there is no vaccine commercially available against both pathogen that causes glanders and melioidosis Worldwide.

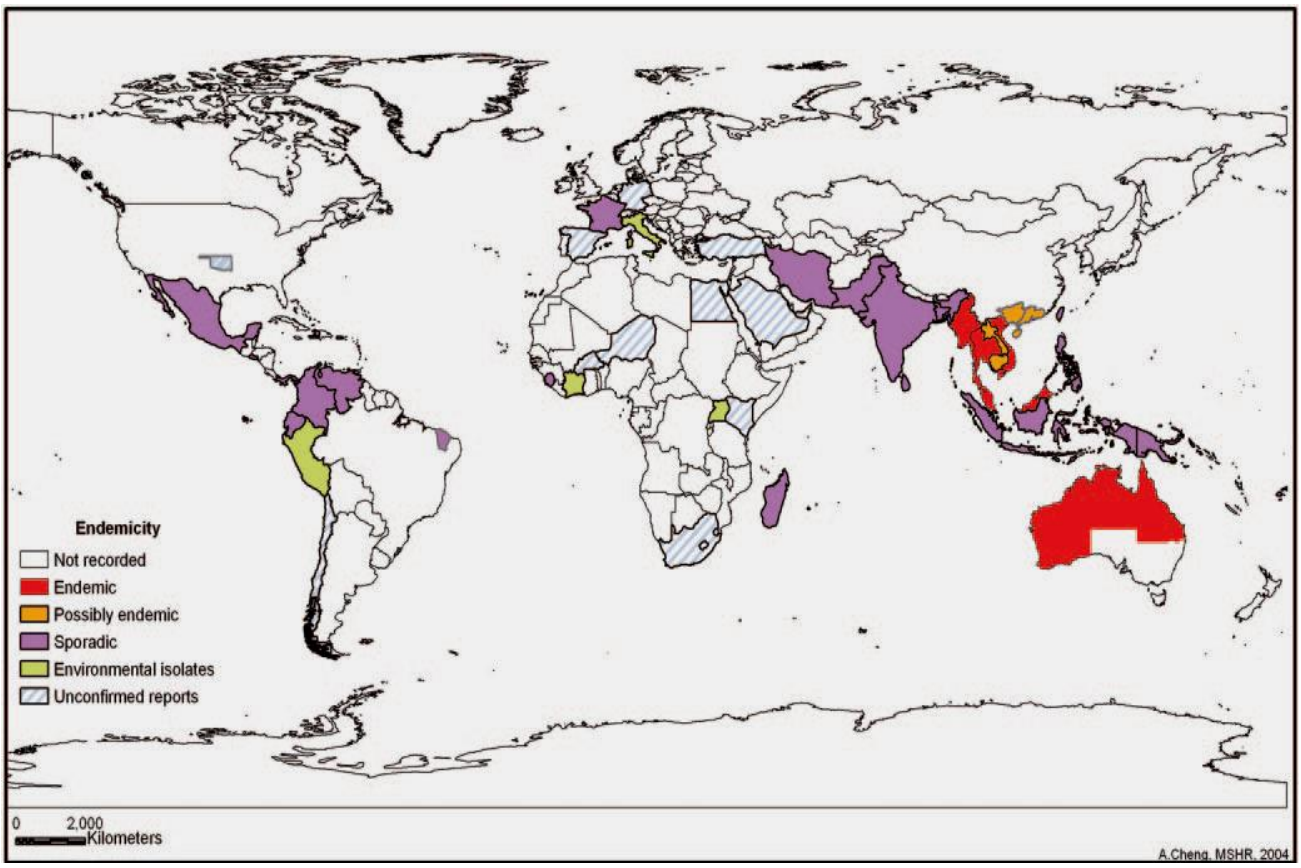

Figure 5: Global distribution of melioidosis. [cited 2008 Jul 14]. 2005; Data adapted from Currie and cheng . Available from: <http://cmr.asm.org/cgi/reprint/18/2/383.pdf>

2.3.1 Infection, Epidemiology and Transmission

The facultative intracellular bacterium, *B. pseudomallei*, that causes melioidosis, is a potentially fatal disease of humans in endemic regions, it affects domestic and wild animals, which, like humans, acquire the diseases from inhalation or contaminated injuries (BOURRIER. 1978, DANCE. 2001, CHENG and CURRIE 2005) The infection can range from acute fulminating septics to a subclinical form that can be identified only by seroconversion in an infected individual. Although it is known that *B. pseudomallei* is an environmental saprophyte, still little is known about the factor that allows to persist, or even proliferate, in some areas and not in others, even though soil characteristics and the climate are clearly important (DANCE. 2001, CURRIE. 2003).

Melioidosis is usually a systemic infection, although some patients present with localized cutaneous or subcutaneous abscesses or lymphadenitis. Systemic infection is associated with abscesses commonly in the lungs, liver, spleen or muscle. Prostatitis and joint infections may also occur (WHITE. 2001). Cases of neurological melioidosis "brain stem encephalitis" were also reported (HASSELL. 2001). Susceptible individuals have underlying conditions such as diabetes or renal disease (CHAOWAGUAL et al. 1989; WOODS et al. 1993). Another risk factors associated with melioidosis include alcohol intake and occupational exposure (GUARD et al. 1984, RODE. 1981), Kova consumption (CURRIE et al. 2000). Case studies in Northern territories of Australia indicated that 85 % of the cases were recorded in "wet" season (ASHE. 2001).

The clinical presentation of melioidosis is similar to that of Gram negative septicemia that misleads primary diagnosis. In humans several distinct forms, ranging from subclinical illness to an overwhelming septicemia, with a 90 % mortality rate and death within 24-48 h after onset are recorded. The disease can reactivate years after primary infection and result in chronic and life-threatening disease (WALSH et al. 1995). The mean time to detect the growth of *B. pseudomallei* is 23.9 h and an increased fatality (73.7 %) was recorded if bacterial growth was positive within 24h compared to 40.9 % if the growth was detected beyond 24 h (TIANGPITAYAKON et al. 1999).

The incidence of the disease in many Asian countries is probably greatly under-estimated mainly due to the lack of suitable microbiological facilities to allow correct diagnosis and corresponding treatment (DANCE. 1991). It has been estimated that the disease incidence in Ubon Ratchathaani in northeast Thailand where suitable laboratory facilities and experienced microbiologists are available, is approximately 4.4 per 100, 000 per year (SUPUTTAMONGKOL et al. 1994). In this province melioidosis is the cause of 20 % of all community-acquired septicemia cases, and 40 % of the deaths (CHAOWAGUL et al. 1989). That shows that scarcity of expertise and medication lead, to higher mortality. The main endemic foci of melioidosis are in Southeast Asia and northern Australia (TAN and GOB 2001, JACUPS and CURRIE 2001, WARNER et al. 2001) where it is a major cause of community acquired septicemia and pneumonia, and has a case fatality of up to 70 % (CHAOWAGUL et al. 1989, CURRIE et al. 2000). *B. pseudomallei* is widely distributed in many tropical and subtropical regions. Epidemiological studies in Thailand and Australia have confirmed

that melioidosis is a disease that presents in rain season in people who have regular contact with soil and water, but they have only been able to identify the mode of infection in 5-25 % of the cases (DANCE. 2001). A survey of melioidosis in farm animals in northern Queensland showed that 75 strains of *B. pseudomallei* were recovered from 30 pigs out of 110; 9 out of 39 sheep; 5 out of 10 goats; 2 out of 8 native birds; one out of 8 horses and one from tree kangaroo through 5 years of investigation (THOMAS. 1981, BOURRIER. 1978). In addition to the livestock species and domestic pets, melioidosis was also confirmed from exotic animals in a Wildlife Park in the Northern Territories of Australia. These include a spider monkey *(Ateles sp.),* gibbons *(Hylobates lar),* a puma (*Felis concolor)* and a cotton-top tamarin (*Saguinus oedipus) (*MILLAN et al. 2001). For the first time melioidosis was also reported among dolphins at an oceanarium (KINOSHITA et al. 2001), that indicates *B. pseudomallei* has a wide ranges of distribution from soil to water and from human to domestic and wild animals.

Melioidosis is categorized as an emerging global problem, as defined by the US Institute of medicine edited by LEDERBERG et al. (1992). Emerging infections are those whose incidence in humans have increased within the past two decades or threaten to increase in near future. Incidentally this definition is from a physician point of view that refers to human only it excludes the role of farm and wild animals.

The organisms may spread to man by invading the nasal, oral, and conjunctival mucous membranes, by inhalation into the lungs, and by invading abraded or lacerated skin. Vertical transmission at childbirth and sexual transmissions have also been reported (LUMBIGANON et al. 1988, 1995, ABBINK et al. 2001). Aerosols from cultures have been observed to be highly infectious to laboratory workers and aerosol spread is efficient and there is no available vaccine or reliable therapy at present (GREEN and TUFFNEL, 1968, MCCORMICK et al. 1975, CHENG and CURRIE 2005)

2.3.2 Multidrug resistance: Unrealiablity up on Antibiotics

The causative agent of melioidosis is characterized to be difficult to treat. The organism is intrinsically resistant to most of the antibiotics tested in vitro and in vivo; it is a slow respondent to drugs and relapses even to drugs to which it is sensitive in vitro and relapses are common after apparently successful primary treatment (TRIBUDDHARAT et al. 2001, CHAOWAGUAL. 2001, VORACHT. 2000, KEITH et al. 2001, BURTINICK and WOODS 1999, BURTNICK et al. 2001). *B. pseudomallei* is resistant in vitro to penicillin, amino-penicillin, first and second generation cephalosporins, most amino-glycosides, β-lactamas, most macrolides, rifampicin and polymyxins (DANCE et al. 1989b, LEELARASAMEE and BOVORNKITTI 1989). Most of the *B. pseudomallei* isolated from 15 patients in Taiwan were intermediate or resistant to ampicillin, flomoxef, cefepime, aztreonam, amikacin, and ciprofloxacin (HSUEH et al. 2001). *Burkholderia spp.* are able to survive within phagocytic cells (PRUKSACHATVUTHI et al. 1990, VORACHIT et al. 1995a). They can produce glycocalyx, and from microcolonies in infected tissue (VORACHIT et al. 1995b). Since

β-lactamas do not penetrate intracellular sites and kill non-multiplying bacteria, treatment with β-lactamas may not prevent future relapse of melioidosis. β-lactamas with good in vitro activity also fail in the treatment of other intracellular infections such as typhoid and legionnaire's disease (MCENIRY et al. 1988). Therefore, the ideal antimicrobial agents for melioidosis therapy should have bactericidal effect, should be able to penetrate phagocytic cells, and eliminate or inhibit the production of a glycocalyx.

2.3.3 Potential as Biological Weapon

Two pathogenic species of Burkhoderia, *B. mallei* and *B. pseudomallei* are studied by different states to be used as biological warfare. They belong to category B of the list of 'Critical biological agents' of the Centers for Disease Control and Prevention, USA (ROTZ et al. 2002). In contrast, to CDC, these pathogens are ranked in the lower half of the group A of possible bioterrorist use by Russian experts (KONDRIK et al. 2003, VOROBEV. 2001). They have a number of features that makes them an attractive candidate for deliberate release including: ability to cause severe, rapidly fatal invasive infection, initiate infection via aerosols, inoculation and possibly, ingestion and intrinsic resistance to many antibiotics, wide range of natural reservoir (HPA. 2003, CHRISTOPHER et al. 2001). Although there no records on *B. pseudomallei* utility as BW, potential risk of *B. pseudomallei* is uncertain, recently it has been considered an important potential bioweapon, with increasing research activities to figure out virulence factors, pathogenesis and to develop vaccine (CHENG and CURRIE 2005, SODEMAN. 1994).

During World War I Germany's programme of biological weapons used the bacteria that cause anthrax and glanders, with series of attacks on horses and mules destined in Europe to conduct covert operation against the United States and FSU (WHEELIS. 1998, GEISSLER. 1998). The plan was to infect large numbers of horses and mules, which should have an effect on troops and supply convoys as well as on artillery movement, which were dependent on the animals. The intention was both, the destruction of livestock and the transmission of the highly contagious, lethal agent from livestock's to human. Human cases in Russia increased with the infections during WWI. It is considered as the first attack with biological weapons in the 20th century (SRINIVASAN et al. 2001). The Japans deliberately infected horses, civilians, and prisoners of war with *B. mallei* at the Pinfang (China) Institute during WW II (HARRIS. 1997). The former Soviet Union is believed to have been interested in *B. mallei* as a potential BW agent after WWII. The scientist in Obolensk succeeded three key areas: Producing a drug-resistant strain of the glanders bacillus (DOMARADISKIJ. 1998, 2006, ALIBEK. 1999). The U.S. army studied this agent as a possible Biological Weapon (BW) in 1943-44 but did not weaponize (USAMRIID'S. 2004). Other countries with a military interest in *B. pseudomallei* included the USA and possibly Egypt (SHOHAM. 1998, HPA. 2003).

The collection of Burkholderia strains in Russia is, said to be one of the most diversified in Europe with both endemic strains isolated in Russia and hundreds of strains from South-East Asia and Europe (ALEXY et al. 1996). It is one of the most studied pathogen in Russia next to Plague. From

the year 1990- 2004 alone, 70 % of the publication of the Anti-Plague Institute focus on Burkholderia species, that characterises a remarkable competence on the pathogenic Burkholderia species (WESTERDAHL and NORLANDER 2006).

The low transmission rates of *B. mallei* from animals to humans is exemplified by the fact that in China, during World War II, thirty percent of tested horses were positive for glanders, but human case from infected horses were rare. *B. mallei* exists in nature only in infected susceptible hosts and is not found in water, soil, or plants as its very closely related species *B. pseudomallei* (USAMRIID'S. 2004).

B. pseudomallei holds a significant public health concern due to its ubiquitous nature and morbidity and mortality in humans and livestock such as sheep, goats and pigs. In addition, the potential of zoonotic transmission from contaminated milk or carcasses has a considerable economic impact. A wide range of animals (terrestrial and aquatic mammals, birds and fish) are know to be affected by melioidosis with varying severity and clinical manifestations (JODIE et al. 2000).

As human and animals are increasingly transported or moved around the world, there are more opportunities for melioidosis to cross state borders and establish new endemic regions. It is even possible that soil and water contaminated with *B. pseudomallei* may be imported from endemic areas to non endemic areas (GALIMAND and DODIN 1982, CDC. 2005).

3 MATERIALS AND METHODS

These pathogens that cause Plague, glanders and melioidosis were considered as the weapons of the future—organisms produced in laboratories and unleashed on unwitting populations to reproduce, spread, and kill. They are as deadly pathogens , much cheaper to create, and much easier to distribute—inside a warhead on an intercontinental missile, in an aerosol can sprayed in a crowded building, or by a crop-duster flying over a major city. Exposure occurs without warning. Infection from only a few minute particles can mean a ghastly and painful death. The biological bioterrorism, the effect of man or natural-caused epidemics are becoming more real.

The FSU was known as “enclosed world of microbial science” in Western hemisphere Research publications in Russian language and Russian scientific journals rarely known in English language publications. Although the agents that causes plague, glanders and melioidosis were studied for biological weapon interest during the cold war area in both East and West blocs, the strength, secrecy and intensity of research has no comparison. As a result, currently what's more known about these three pathogens is predominately published in Russian language and Russian scientific journals, which are rarely known in English language scientific journal publications. In cases where they were/are cited, they are mostly “incorrect”, misinterpretations or misunderstandings are frequent.

Thus these dissertation attempts to evaluate, analysis, compares the original Russian language published academic journal and the exceptionality of research achievements of former Soviet Union and now know as Commonwealth of Independent States. I am privileged in having an opportunity to study and work in both Russian and English language that enabled me to take this complex hard work, which is not only of Public Health but, also Security, Political, Economical, not less Psychological impact at global level.

To better understand the frameworks, the materials used for deriving the necessary and relevant data to reach an argument are based on an extensive searches of published and unpublished literatures and academic journals written in Russian language compared to English language at the subject coincides. Unique results for which no comparable studies are available, the research results are analysed according to its relevance to the subject and its significance for future perspectives of developing any types of vaccines against the pathogens in concerns either classical forms or recombinante protein basis. The analysed items in this work are accessed in using the following forms of methods of information search:

1. International Congresses, Conferences, Workshop Handbooks, and Abstracts

In Proceedings of the Scientific and Practical Conference dedicated to the centenary of the Russian Anti-Plague service held from 16th - 18th of September 1997, Saratov, Russia. Two volumes of the delegate hand books and abstracts were provided from Professor Dr. Dr. Andreas Hensel, the president of federal institute for risk assessment, Berlin, Germany, Dr. Heinrich Neubauer, head of the institute of bacterial infections and zoonosis, deputy of the president of the Fredrich Loeffler Institute, Jena, Germany and Dr. Ernst-J Finke, the head of the institute for microbiology, German Federal Armed Forces, Munich, Germany. The conference summed up its meeting with two volumes of delegate books and abstracts in Russian language in 466 pages. Volume 1 consists of the epidemiological and immunological section of the conference and Volume 2 contains microbiology and diagnosis part. As the title of the conference indicates, the Anti-plague system, in its kind showed a long year of research activities and achievements in fighting infectious diseases. Relevant data related to the topic of the dissertation are thoroughly analysed and cited in this work.

The delegate handbook and abstracts of world melioidosis congress incorporating the inaugural emerging infectious diseases of Indian Ocean Rim (EIDIOR) workshop, that took place from 26th - 29th of September 2001, Perth, Western Australia, was kindly posted to us from Dr. David DeShazer, bacteriology division, United States Army Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA. The first 21 papers presented on September 27th summarise epidemiological, clinical, diagnostic, antibiotic resistance and treatment aspects, and 26 papers presented on September 28th includes pathogenesis, Veterinary and molecular epidemiological aspects of *Burkhoderia* species. The Handbook consists of 66 pages.

Public workshop on animal models and correlates of protection for plague vaccines, that took place on 13th of October 2004 in Gaithersburg, Maryland, USA, was kindly sent to us from Professor Andery Pavlovich Anisimov, The director of the Department of Infectious Diseases, State Research Center for Applied Microbiology, Obolensk, Russia. The content of the document is compiled in 222 pages, which describes the current state of knowledge on plague pathogenisis, animal models, new vaccine development in western scientific research approaches.

2. Scientific Journal published, Abstracts, Summaries or Titles

An extensive survey of published and unpublished literatures written in English language was carried out both through use of multiple search machines, based on electronic database like pub-med, science, nature and other available life science domains. In case the relevant items are not available in electronics form, then traditional paper obtaining, i.e., subscription of the article to acquire the copy through library loan procedures, mixed–mode and email contact to the Authors were used.

In order to obtain items, which were relevant to the topic, the following key words are used to identify the articles availability in electronic (Internet) database: Plague, *Yersinia pestis*, Vaccine, Glanders, *Burkholderia mallei*, Melioidosis, *Burkholderia pseudomallei*, Immunogenicity, Virulence, Genetics, Biological Weapon, Soviet Union, UDSSR, Russia, Anti-Plague Institutes alone, and combined as it fits in [MeSH] browser, the National Library of Medicine Database. In addition, "the related links" options were used to the identified article accessibility.
For some relevant historical documents available in electronics form, some fully and some partially scanned items like books, reviews, Public-workshops, Congresses-notes, meeting, interviews, newspapers, comments, video/ audio data and forums relevant to the topic are used to retrieve the data.

3. Scientific Journal articles published in Russian Language

Although Russian language written scientific publications are rarely available in World Wide Web search engine to retrieve some of the available documents relevant to the topic the Cryllic (Russian) Alphabets and the corresponding Russia terms to the English language were applied as keyword, for instance instead of plague The Russian term; "Чума," and instead of Glanders the Russian term; "Сапа," instead of Melioidosis the Russia term "Мелиодоз," instead of Vaccine the Russian word for it "Вакцин", e.t.c. As most of the article published in Russian language, and Russian scientific journals were/are not available in electronics form, mixed-mode subscription of the article to acquire the copy through library loan and email contact to the Authors and Institute Directors of the Anti-Plague Institutes were implemented. These methods were the main and most efficient way of obtaining relevant data sources included disclosure forms and official documents maintained by the Anti-Plague Institutes in FSU and current state of research activities in Russia on the particular pathogens in concern.

4. Books

Cited books or its parts relevant to the topic were subscribed according to National and International Library loan procedures. Cited sections were either copied or thoroughly studied and informations were extracted in case the book was at hand. The other options of obtaining were books out on loan from Library.

5. Dissertations and Theses

Dissertations and Theses, titled as [Major criteria of evaluating new vaccine strain of plague microbe, enlightenment of Methods] published by the Ministry of Health of Russian Federation State Institution of Standardization and control of medical and immunological preparations in the name of L.A.Tarasov, Russian research Anti-Plague Institute "Microbe", Research institute of microbiology of the Ministry of Defence of Russian Federation. The document was compiled with 70 pages, describing all requirements of new vaccine candidates of plague vaccine. And the other Theses titled as [Molecular-genetics of Capsule formation and function of *Y. pestis*] which was compiled with 272 pages were kindly sent to us from Professor Andrey Pavlovich Anisimov, Head

of the Department of Infectious Diseases, Russian State Research Center for Applied Microbiology, Obolensk, Russia.

6. Email contact to Authors

Personal Email communications were made to obtain relevant documents, which were not accessible in other methods described above. These documents were kindly sent to us from Professor Piven, Volgograd Sceintific and Research Anti-plague, Volgograd, Russia, publication in concerns of glander and melioidosis, and Professor Anisimov, Obolensk, Russia in concerns of plague.

The Anti-Plague Institutes of CIS have long period of research activities, resources, experts and unique records on plague, glanders and melioidosis diseases. For example, alone from the year 1985-2004 the Volgograd API has published more than 120 scientific research articles on *Burkholderia* species causing glanders and melioidosis written in Russian language and published in Russian scientific journals, which would be thoroughly analysed in this work.

Although the title of the dissertation is self-explanatory for most parts of the work concerning the time frame, "the former Soviet Union" it also includes the current state of knowledge of vaccines under trail in the region on the pathogens in concerns and the magnitude of gene manipulated infectious disease of group 1 BW interests.

The criteria for evaluating the available materials in this work was based on the properties of Effective vaccines for prevention of diseases caused by potential bioweapon / terrorism agents like plague, glander and melioidosis. A vaccine that can provide universal protection, acceptance to the neutral bodies like WHO, UNICEF, and manufacturers and global utilization for the whole ranges of population in need. Currently there is no particular position on Plague, glanders and melioidosis from WHO, as there are announcements of WHO positions for some other vaccines. Thus, the basical requirements for a vaccine against the pathogens in concerns for human use should give, 1) life-long immunity, 2) be broadly protective against all variants of an organism, 3) prevent disease transmission, such as shedding, 4) induce effective immunity rapidly, 5) be effective in all vaccinated subjects, including infants and the elderly, 6) requires few (ideally one) immunizations to induce protection 7) be stable (no requirement for cold chain, 8) safe, 9) cheap, 10) sustain confidence in people (WILLIMASON. 2001, BEVERLY. 2002, HASSANI et al. 2004, ANISIMOV et al. 2004, WHO. 2007, FEODEROVA et al .2007, 2008).

Thus, analyses of research developments on pathogens in concerns were made according to these criteria and the extent of vaccine type in fulfilling these requirements, Although few real vaccines approach this goal, It is the theoretical aim of any research and development of vaccine to achieve these goals in using modern molecular tools from which development of vaccines may possibly profit. The strength and weakness of vaccines studied, single or recombined protective antigens are thoroughly analysed according to the above-described properties of the vaccine type and the

current state of knowledge of vaccine in one side and the immunology of the host and host–pathogen interaction on the other side.

Documents found from data base archives, describing the backgrounds of varies research approaches made to develop vaccines against, *Y. pesits*, *B. mallei* and *B. pseudomallei* are briefly described according to their stages of developmental chronicles, their efficiency, and their role in the past and impact on the future studies in attempts to develop new or improved vaccines based

4 DEVELOPMENT OF VACCINES

Indeed, vaccines are considered among the most, if not *the* most, cost-effective and successful approach in medical development to prevent infectious diseases, because they have successfully eliminated an entire wild type disease from the planet Smallpox, (WHO. 1979) with a second disease about to eradicated (Poliomyelitis) and control of several different infectious diseases including measles, mumps, rubella, tetanus, diphtheria, pertussis, *Haemophilus infleuenzae* type B disease e.t.c. (WHO. 2004). The secret behind this success lies to a large extent in the ability of the vaccines to teach the body to respond to the wild pathogen, rather than directly treating the disease, as therapeutics such as antibiotics do. Mortality and morbidity due to several infectious disease have been dramatically affected by wide spread use of immunization (WHO,199, 2000). Yet there are dangerous pathogens like *Yersina pestis* renowned for being the agents of the Black Death and is one of the most potent bacterial pathogens feared that can infect human for no vaccine is available in western hemisphere or the agents of glanders and melioidosis for not yet developed. The availability of these pathogens around the world, manifestation of multidrug resistance strains, capacity for mass production and aerosol dissemination, difficulty in preventing high fatality rate of aerogenic infection and potential for secondary spread of cases during an epidemic in case of plague, and the potential use of these pathogens as a biological weapon enforces the development of an effective vaccine as the most appropriate and beneficial measure to combat against these pathogens that have a great Public health and security concerns (STEPHEN,1995, GALIMAND. 1997,RASOMAMUNA, et al.1995, EVECHENKO et al. 1997,INGLESBY et al. 2000, AMPEL.1991).

Although our knowledge of understanding the pathogens to genomic level, host-pathogen interaction, efficiency of the protections and virulence of expressed single or recombined antigens and to some extent their mechanisms and induction of the Immune systems mediated in the host in the past two decades, currently, there are some significant roadblocks to the development and licensing of improved or new vaccines, that includes biological and technological issues, but to the large extent, the major challenges are the difficulty in preparing a 100% safe, efficient product, for universal application and the high cost of testing and almost unavoidable consequences of the occasional, no matter how frequent and how minor adverse events are reported (ROSEENTAL and ZIMMERMAN 2006).

4.1 Plague Vaccines Licenced for Human use

4.1.1 Killed Whole Cell Vaccines

The first plague vaccine was named after Haffkine. It was developed from a heat–killed, old boullion culture in 1897, three years after Yersin identified the causative agent of plague. It was widely used to fight against plague in endemic regions of the world such as in India, Egypt, Uganda, Kenya, Madagascar, Java, Senegal and in some natural plague foci regions at that period (DESMIDT. 1929, ECHENBERG. 2002, ISUPOV. 2002). It was administered subcutaneuosly in a dose ranging from 2.5 to 12 ml per inoculation varying in methods of its production (TAYLOR. 1933). Such as, vaccines produced in Nairobi, Kenya; by methods of Haffkine was inoculated subcutaneous in a dose of 2 ml. In the year 1914, alone in Senegalese epidemic outbreaks, about 62,776 persons were immunized with Haffkine vaccine. The injection was invariably given subcutaneously on the arms of the patients. When abdominal injection was attempted, not only did the patient object, but also it was found that belts rubbed against the mark produced painful abscesses. Later on, it was observed that the vaccine produced a wide variety of side effects ranging from more soreness at the point of inoculation and low level of fever to painful swellings of the lymphnodes in groin or armpit, where some of them where persisting for a month or more (ECHENBERG. 2001). Breast-fed children were found to have vomited the night following the inoculation of their mother. Furthermore deaths of immunized individuals was recorded, that led to the rejection of further vaccination by the population. Similar problems were observed in Kenya, Hawaii where Haffkine vaccine was used against plague epidemy (DESMIDT. 1929). In addition to the vaccine, Haffkine also worked on methods of preparations of anti-plague serum, gained from horses and used as prevention and treatment of individuals living in natural foci regions and for those who had contact with patients voluntarily (HAFFKINE. 1897).

At the beginning of 1930s epidemiological investigation of the regions where Haffkine and other similarly killed-vaccines used, were proofed to be of risk to prevent from the epidemy of plague, especially in areas where they were applied earlier, like in Madagascar, Java and others (WILLIAMS et al. 1980, MEYER. 1974). This phenomenon was explained differently: some argued that it is the result of the denaturation of the immunogen part of the plague microbes, the bacterial protein that induces antibody and other authors argued that the killed vaccine lacks the V-antigen, one of the major immunogenic proteins of the pathogen. That being the general view of that period, today it is known that *Y. pestis* harbors in addition to the two known major immunogenic antigens, the F1 (Fraction 1) and LcrV (Low cacium response Virulon), additional groups of highly conserved proteins designated as YOPs, which are essential virulence factors of the pathogen, and some of them are also known to be immunogenic (CORENEILS. 2000, TROSKY et al. 2008, VIBOUD and BLISKA. 2005). *Y. pestis* also harbors an antigen called Pesticin (BEN-GURION and HERTMAN 1958, BEN-GURION and SCHAFFE RMAN 1981).

During the decade preceding World War II, research on killed plague vaccines was at low ebb. In the United States, however, studies on killed vaccines continued in the laboratories of the Hopper Foundation, University of California which was known as United States Army Vaccine (USP), a formaldehyde-killed preparation of the highly virulent strains like Shasta, Yerka and 195/P strain of *Y. pestis* that replaced the Haffkine vaccine (MEYEER. 1964, MARSHALL et al. 1974). Although preliminary experimental results established in animal models, differs from authors to authors in its level of protection, such as killed plague vaccine afforded more than 90 % of protections for guinea pigs against s.c. infection dose of 1.8×10^{10} CFU of *Y. pestis* was reported by MEYER (1970), and in contrast CHEN et al. (1974) experimental studies found that killed plague vaccine showed only 40-75 % protection of guinea pigs against 10^6 and 10^3 cfu challenged s.c. with virulent *Y. pestis* strain 195/P.

In case of the clinical responses of the vaccine in humans showed an alarming effect: fever, malaise, myaligia, dizziness', gastrointestinal symptoms, insomnia, persisting for weeks. Based on long years observation, October 1941 the subcommittee on Tropical Diseases, National Research Council committee on Medical Research, USA, passed the following resolution on plague immunization:
"Resolved that, even the available knowledge does not seem to afford definite evidence of the benefit of the use of plague vaccine, it is considered advisable to vaccinate with killed plague bacilli of an approved strain all military or naval personnel under serious threats of exposure to bubonic plague."

Based on this recommendation, vaccination with killed plague vaccine was initiated in the armed forces. There were varies modified batches of USP vaccine, designated as Vaccine A, produced between 1942-1945, based on Shasta strain of *Y. pestis* culture, consisting of $3.6\text{-}4.0 \times 10^9$ cfu, Vaccine B produced from 1950-1967 based on Yerka strain of *Y. pestis* containing 2.0×10^9 cfu, and vaccine C which was produced since 1967 to 1999 based on *Y. pestis* 195/P consisting of 2.0×10^9 cfu. in USA. All these vaccines were used in immunization of human and thoroughly studied by MEYER and MARSHALL et al. (1974). Generally, all of them (A B C) showed general and systemic adverse reactions, no evidences of protection for pneumonic plague (RUSELL. 1995, WILLAMSON.2009).

Summing up all kinds of killed plague used in human in the past, beginning from Haffkine vaccine to USP vaccines , basically, they all consists of heat or chemical killed bacterial cell cultures of different virulent strains of *Y. pestis*. Even though methods of killing and immunization schedules are slightly different between them and there are various bits of evidences that killed whole cell vaccines do protect against bubonic plague. But, conversely there is no evidence of protection against pneumonic plague. There are numbers of evidences that showed experimental animals immunized with killed vaccine developed pneumonic plague. Similar cases, where people have been immunized with killed whole cell vaccines have contracted and developed pneumonic plague.(DOLL et al. 1994, TITBALL and WILLIAMSON 2001, 2004, WILLIAMSOM .200). There is also no evidence of protection caused by virulent atypical strains of *Yersina pestis* which are isolated from natural foci and know to be virulent (ANISIMOV et al 2004).

The other issue of concern with killed vaccines were/are their reactogenicity, side effects following immunization, short term protection, that frequent revaccination, (3 booster immunization per year),
requires multiple immunizing doses and short period of protection. Vaccine production needs especial containment, they are all expensive and have low public-confidence. There is no evidence of protection against infection caused by atypical strains known and isolated from natural foci regions. Killed Plague vaccines are unable to induce all desired types of immunity to clear up the pathogen. and do not fulfil the current standard of vaccinology, immunology, biotechnology and the requirements which the authorities like WHO, UNICEF, or any vaccine producing manufacture (Tab.1) needs to produce licence the vaccine for human for global application. From immunological point of view, the main drawback of killed vaccine is that it cannot mimic the steps and processes elicited during the establishment of the natural immunity which is ultimately controlled by cell-mediated-immunity that requires a robust and protective T-cell memory response to clear up the pathogens from the host organism, as *Yersina pestis* is a facultative intracelluar (CORNELIS. 2000, SMILEY. 2007, WILLIMASON,2009).

The KWCV is not more available for human uses, the manufacturer discontinued producing it in 1999. Currently, the only killed whole cell vaccine, which is available, is the vaccine produced by the Commonwealth Serum Laboratories in Australia (CORNELIS. 2000), and is reported to be for research purpose.

Table 1. USP Vaccine compared to Ideal (Effective) plague vaccine

Descriptions	USP	Ideal(Effective) vaccine
Protection	Only Bubonic	Must protect all forms
Duration	Short term	Life-long protection (Ideal)
Universality	limited	Must protect from all variants
Side effect	High (MSD 12%)	Zero tolerance (Ideal)
№ immunization	Short term 3 booster inoculation	One inoculation, induce effective immunity rapidly
Stability (Cold chain)	Stable, but requires cold chain	Stable no requirements of cold-chain
Safety/Handling/production	Special containment	Not needed
Cost	Expensive	Must be cheap
Public confidence	Negative	Must sustain confidence
Induced Immune system	Humoral	Humoral and cellular Immunesystem

USP- United States Army Plague Vaccine, MSD-Mean standard Deviation, Data adapted and compiled from (Rusell et al. 1995, Myron. 2000, Meyer. 1974, Marshall. 1974, WHO. 2008, Beverly. 2002,WHO,2005).

4.1.2 Live Attenuated Vaccines

Historically the first trail of live attenuated vaccine was Yersin himself. He was quite interested in determining which species most closely reflected human. Therefore, he isolated a live attenuated vaccine strain, which he found virulent in rats but not in five species of macaques and injected himself. Luckily, he survived (YERSIN. 1900).

The first experimental studies on humans demonstrated by STRONG. (1908) that live attenuated vaccine was evidently superior to others like heat or formalin killed vaccines. They provided a great degree of protection. He observed that the experimental animals obtained higher immunity as when they were immunized with live attenuated vaccines. For instance, monkeys which had been immunized against plague by live attenuated vaccines were immune against multiple subcutaneous inoculations of lethal doses of virulent strains of *Y. Pestis.* Further strain like *Y. pestis* Tjiwidej were also tested in humans, and were considered to have been highly effective in reducing plague morbidity on the islands of Madagascar and Java. It was expected to be 90 % effective in areas where it was used (GIRARD. 1963). Vaccines derived from live attenuated organisms are known for inducing cellular immunity efficiently that provides protection against intracellular organisms like *Y. pestis*, however mild allergic reactions of local form in 4.2 % of the cases and 3.7 % general reaction were reported following subcutaneous inoculation of 10^9 organisms, and

similar results were achieved during mass vaccination in South Africa (GRASSET. 1942, 1946) and Senegal (ROTMAN. 1945). Another live attenuated plague vaccine of EV strains initially isolated in 1926 from human cases of bubonic plague in Madagascar and then attenuated by continuous *in vitro* passages on nutrient medium at the room temperature (18 °C-25 ° C) for 6 years were used (GIRARD. 1936). Different lines or subcultures of the EV strains were used as live attenuated vaccine to combat plague worldwide (GIRARD. 1963, MEYER. 1970). One of the most frequently used derivatives of the EV strain is the *Y. pestis* EV76 live vaccine.

In the former Soviet Union studies on EV 76 live vaccine strain proposed by Girard and Robic for vaccine production against plague began in 1936 and the most common derivatives frequently used for human vaccine is *Y. pestis* EV line NIIEG (designated based on the Russian abbreviation of the Scientific- Research Institute for Epidemiology and Hygiene, Kirov, Russian Federation) where the vaccine was maintained for long period of time. Also, in both NIIEG and the Russian State Anti-plague Research Institute 'Microbe` Saratov, Russia. The major protective and immunobiological properties were studied, methods of optimizations and techniques of lyophilization in special dry nutrient medium were developed (FAIBICH et al. 1947, CHALISOV. 1947). After initial vaccination of more than seventy thousand volunteers the vaccine showed relatively low frequency of adverse effects and high protective efficiency, thus it was recognized for live plague vaccine production and immunization for human(GIRARD.1935, SALTYKOVA and FAIBICH 1975].

The drawbacks of formaldehyde, heat killed vaccines or fractions of plague microbe (F1) in experimental animals were intensively studied in the FSU (KOROVKOVA. 1955, 1957, 1962) that led to find another methods of attenuation, such as heating, vaccine strains, like the *Yersina pestis* of EV 76 cell culture grown on Hottinger agar medium in the presence of highly concentrated Saccharose solution was developed. This method was expected to reduce the intensity of the denaturation of immunogenic substance of the vaccine strain. The first lot of vaccine produced intended for human in this method was known as AD-Vaccine. The vaccine was used from the, during the campaign of eradication of plague in Volga-Ural natural foci in Gureiv region year 1941-1942, where 40,143 people living in the region were immunized. Although the vaccine better protection was seen when compared heat or formalin killed vaccines used prior, it was not produced and used in human further. In 1943, it was replaced by the live attenuated EV 76 strain line NIIEG (OSOLINKER. 1960, ISUPOV and LEDVANOV 1997, 2002, SALYTKOV and FAIBICH 1975).

In 1940, the institute of "Microbe" organized and performed a production of a vaccine in a liquid form. Correspondingly, the NIIEG studied major morphological, biochemical characteristic, stability of the vaccine strain, developed methods of optimizations, lyophilization techniques in special dry nutrient medium of more stable form of live-dried plague vaccine that retains its activity for not less than 1 year (FAIBICH et al. 1947, CHALISOV. 1947) . Relatively low post vaccine adverse reactions were seen in immunization studies made on more than 66,000 people (KOPILOV et al. 1947). Furthermore epidemiological efficacy of the vaccine was also studied during an eradication programme of epidemic outbreaks as a consequence of Japanese bioweapon

experiments during World War II in Manchuria in the year 1945-1946 (SALTYKOVA and FABICH. 1975, FEODOROVA et al. 2007, 2008). Millions of Soviet Red Army personnel's were immunized during this period under physically difficult conditions and there was no single case of infection reported from the vaccinated personnel's, where there was high infection rate in the region and the inhabitants (DOMARADISKIJ and OREINT 2006).

Different lines of subcultures of EV76 strain were employed as live plague vaccines world wide, resulting in considerable reduction of mortality and morbidity of human plague (FEDEROVA et al 2007). However, there were reports from different laboratories, that EV76 vaccine strain might posses residual virulence in mice and other animals species (MEYER 1970, RUSSELL et al. 1995, TITBALL and WILLAMSON 2004, HALLET et al.1973), raising a question of "residual virulence" which might occasional cause disease in human post vaccination and its widespread use might be precluded by practical constraints such as manufacturing and safety concerns. Live attenuated vaccines needs systematic investigation of the vaccine strains, beginning from its quality, stability, biochemical and morphological characteristics, immunogenicity, reactogenicity, fluctuation during processing, production, storage, and in courses of immunization. Variation of a strain in one of these characters from one to the next generation usually leads to "Saprophytization", results to deterioration or a loss of its immunological values indicating the disqualification of the candidate strain for further investigation for vaccine production. Thus, special experimental investigation were conducted to prove the stability of the EV76 line NIIEG in comparing to various EV 76 cultures donated from Institue of "Microbe", Irkutiskaya Anti-Plague Institute, Robert Koch Institute Berlin, Germany and Institute of Hygiene Dairen, China form 1942-1947 [Saltykova. 1947] cited by [SALTYKOVA and FAIBICH 1975].

Immunological investigations were carried out in guinea pigs. A single subcutaneous Immunization of animals with different EV culture from various institutes followed by infection with highly virulent strains of *Y. pestis* culture were compared for 30 days. These studies confirmed that a culture of EV strain preserved in the museum of NIIEG, showed to be highly resistant against the impacts of various biochemical and physical factors imposed on the strain during the experiment and retained its minimal immunization doses in the limit of $1x\ 10^5$ cfu, capable of inducing higher expression of immunogenicity. Higher immunogen expression was observed from animals immunized with *Y. pestis* EV 76 strain line NIIEG, which was attenuated in 10 % saccharose solution and stored in an ampule at 8°C in a vacuum for five years (CHALISOV et al. 1947, AKIMOVICH et al. 1964,)Alteration of growth on nutrient media, which indicates the reduction of immunogenicity, was observed only after 100 serial passages of the strains through nutrient broth, application of special phages, or UV-radiation (SALTYKOVA and FAIBICH 1975).

Although there were no essential cultural-morphological differences observed between different culture of EV strains compared, and none of them killed guinea pigs and mice challenged subcutaneously with a dose of up to $2x10^9$ cfu but, significant differences were obtained in their level of protection. Cultures received from Institute of "Microbe", Irkutsk Anti-plague Research Institute, Berlin and Dairen showed definitely less protection. Even the highest doses of

immunization (1 x10^9 cfu) followed by infection of virulent strains protected less than 50 % of the animals. According to the Russian state standard, requirements for plague vaccine, a minimal immunization dose consisting of the lowest cell concentration must be able to provide not less than 80 % protection of guinea pigs against challenges with 200 DCl 21-days after immunization (ANISOMOV. 2002).

The high immunogenicity of the live attenuated strain of EV 76 line NIIEG was accompanied with its high survival rate in inoculated animals such as in guinea pigs, that raises the question of Infectiousness, the possibility of uprising of "residual virulence" This question was addressed in a series of experiments conducted in guinea pigs, mice and fleas that showed no signs of reversion or changes of the stability of the vaccine (SALTYKOVA and FAIBICH 1975). As a result, In 1947, It was concluded to be produced as a "Etalon" (National standards of reference) strain for plague vaccine production and to be used as a quality measure to evaluate other candidate strains and cultures to develop new or improved plague vaccine.

The live-dried plague vaccine consisted cells of *Y. pestis* EV 76 line NIIEG ;a culture grown on Hottinger agar medium, washed off with 10 % saccharose solution and lyophilized according to NIIEG methods and stored in ampules at 8 °C under vacuum as "series №303". It was used to produce a vaccine against plague until 1953 (CHALISOV et al. 1947).

From the year, 1953-1959 a "bivalent" dry-plague vaccine from EV strain designated №1 proposed from the Institute of "Microbe", and strain № 17 from Irkutsk Anti-Plague Research Institute were released as a recombinant vaccine known as vaccine №1-17 and 93,289 people living in Volga–Ural natural plague focus were immunized with it, where the recurring epizootic activity of plague were occurred from 1954-1956 (OSOLINKER. 1960).

Experimental investigation of the new vaccine strain № 1-17 was thoroughly studied by KOZLOV et al. (1960) that includes, clinical healthy volunteers of, 136 females and 155 males with an age between 25-35 years, intracutaneously immunized with similar doses of the some serie prepared at the Irkutsk Anti-Plague Research Institute with in two days. The study indicated that the vaccine showed high reactogenicity as well as cultural-morphological and biochemical instability, risks of alteration that can led to virulence. Thus, the drawbacks of vaccine strain № 1-17 were confirmed at the end of 1950s and was not more produced.

Since 1960, the vaccine strain of EV 76 line NIIEG line and its subcultures are used in producing the live-dried-plague vaccine for different routes of administration such as inhalation or oral application (LEBEDINISKII. 1971).

In the year 1971 the WHO committee of expertise recommended to produce live-plague-vaccines to those States who wishes to develop plague vaccine from "*Its' original approved maternal strain of EV, preserved its' properties without any alteration"*. Although the EV vaccine strain was the

strain of choice that fulfilled necessary requirements for vaccine production at that period, no plague vaccine is licenced by WHO yet.

In August 1979 a resolution was ratified by the Ministry of Health of USSR on a national criteria for plague-vaccine testing and in the year 2002 updated by Federal Centre of State Epedemic Surveillance of Ministry of Health of Russian Federation, Moscow, is currently applicable and is known as, Russian national criteria for plague vaccine-testing (the main requirements for evaluating of new vaccine strains of the plague pathogen): Methodological Guidelines MI 3.3.1.1113-02". It consists of 15 headings, subheading and 7 appendices with exact description in 69 pages. Although each line of the statement in this particular document is equally important and one procedure can not executed without the success of the other, here the general regulations and criteria required for candidate strain for the developments of improved or new vaccine compared to the standard vaccine used in FSU/Russia are briefly described.

The introductory part of the document in the 1st heading briefly describes the characteristic of plague microbe and the deployment of live vaccine against plague prophylaxis in Russia. The quaility of attenuated strain of vaccine candidate of choice must be compared to the Etalon (reference) EV line NEIIG strain obtained from State Research Institute of Standardization and Control of Medical Biological Preparations named after L.A. Tarasevich. The 2nd heading describes, norms, references and regulatory authorities, institutions e.t.c. in concern. The 3rd heading describes the general position for the strain of choice for live vaccine production, its stability in losing its' virulence in human, but retains its' residual virulence in mice and higher doses in guinea pigs, investigations must be of bio-safety group III categories. It also determines the number of cells (10 Units= 1.10^9 cfu) to be used, generation (2nd) of culture grow on agar media. The 4th heading describes the identity of the strain of choice requirements at species level, i.e a candidate strain must be lyse by the L-413C plague phage, must show typical cultural-morphological and biochemical properties', must harbour three DNA plasmids *pFra* (60 MD*), pCad* (47 MD) and *pPst* (9 MD) with corresponding molecular masses.

The 5th heading determines the non-toxic character and the extent of residual virulence of the candidate strain, extents of dissimilation, survival of the bacterial cell in the tissue and organs or of guinea pigs, methods and reference standards of the procedure. It also explains the importance of residual virulence of the strain, ability to multiply in the organism, as a result an effective immune system of the host is induced. The strain of choice must be able to replicate and survive in the body of inoculated animal with in 3-15 days, activates the construction of immune system of the host. After 30 days, as a rule the animal is free of bacteria cell. The 6th heading briefly describes similar parameters as heading 5 but in white rats.

The 7th heading describes the extent of the candidate strain in losing it virulence and methods applied. A cell cultured after 10 s.c. serial passages of the candidate strain through the organism of guinea pigs must not show residual virulence, must not alter its' cultural-morphological, and biochemical properties; plasmid profiles.

The 8th heading illustrate methods and norms of determining the reactogenicity of the candidate strain in guinea pigs challenged s.c. Highly immunogenic vaccine strains of plague microbe must be

able to induce acceptable local and systemic reactions related to the concentration of bacterial cell acceptable ranges of body weight lost, mean body temperature raised in the group of animals.
The 9th heading describes morphological indications of strain of choice in guinea pigs following s.c. challenges. The extent of morphological as histological changes of regional lymph nodes, and internal organs (liver, spleen, kidney) of the animal as a results of residual virulence and corresponding method to be applied. This section also illustrate dose, duration, organ related changes and standard references that the strain of choice must fulfil.
The 10th heading describes the reactogenicity of the candidate strain after aerosol (inhalational) immunization in the guinea pigs and methods applied. It determines the time-organ related numbers of bacterial cell accepted when recovered form different organ-homogenate. For instance isolation of single cell from lung, form 1-14 days and regional lymph nodes is acceptable only at this time range. Reactogenicity is evaluated in relation to the average body weight the animal lost in group after inoculation, thus with in 7 days lose of weight (> 5%) and body temperature raised (> 1.5 °C) is not accepted. It also determines types of morphological and histological changes in tissue and organs, which are or not accepted.
The 11th heading briefly describes reactogenicity of the vaccine strain in guinea pigs under stress condition and methods applied. The 12th heading describes the impact of the strain of choice on the immune system of guinea pigs and the methods used. It express that a 1.10^5 cfu s.c. challenges choice must not have a suppressive effect on humoral and cellular immune system; rather must be able to increase the quality of lymphocytes found in $S+G_2+M$ phases of cell cycle from 1.5- 2.0 folds.
The 13th heading describes immunogenic activity of candidate strain. Plague vaccine strain must protect guinea pigs against s.c. and aerosol infection with virulent strain, and white mice from s.c. infection. Thus the strain of choice must efficiently provoke the formation of immunity in the host and durable. It describes the ID_{50} in guinea pigs (< 1.10^3 cfu) and white mice (<1.10^4 cfu).The minimal vaccine dose (5.10^3 cfu: standard reference) must provide not less than 80% and 50 %the animals challenged with 200 DCL of virulent strain 21 and 7 days post vaccination respectively.
An aerosol immunization dose of 2-8. 10^6 cfu of standard vaccine or candidate strain must protect 8 out 10 guinea pigs against plague microbe.
The 14th heading describes the stability of strain in processing and production and the final heading describes a dry plague vaccine trails in human, regulatory norms and authorities (BURGASOV et al. 1979, ANISIMOV et al. 2002).

It was estimated that more than 10 million people living in different states of the world including former Soviet Union were immunized with live-attenuated EV 76 line NIIEG against plague. A vaccine was used as the best prophylactic measure in combating varies endemic outbreaks in the FSU. Furthermore it was also known, that during the war more than 70,000 people were immunized under physically difficult conditions in the USSR alone (KOPILOV et al. 1947).

Based on the proposal of the API experts on the vaccine, in case of a great epizootic outbreak in the population living in natural foci territories of CIS, mass vaccination were necessarily organized. Such as, massive immunization project conducted in 17 endemic regions, where constant epizootic outbreak activities were emerged. There were also additional evidences that confirms, the efficacy

of the live attenuated vaccine of EV-NIIEG line, such as, the investigation that included 7 rural villages, where 33,675 people lived, where there were 2,127 cases of human infection registered through epidemiological surveillances before massive immunization took place and 223 cases were proofed to conduct a disease (SVISTUNOV. 1997).

Another immunization program with the vaccine name known as serial № 22, ОБК № 229 of Stavropol Anti-Plague Research Institute, was also used in human. The program was accomplished within 25 days, which covered 2,524 individuals including children. Children with an age under 6 were immunized with $1x10^8$ cfu and an age of 7 and upward, with $2x10^8$ cfu. As the epidemic broke out, 37 individuals showed sign of infection, where 10 of the patients were not immunized, 1 patient died of "primary septicemic plague," 7 of the patients conducted "bubonic-septicemic plague" and 2 patients presented bubonic plague. 27 cases of the patients who conducted infection belonged to the vaccinate groups (SVISTUNOV. 1997). The reason why the immunized individuals conducted infection is not further explained.

Inhalation and oral vaccine were thoroughly investigated by ALEXANDROV'S and associates' (1962) as a dry dust (aerosol) plague vaccine based on EV strain line NIIEG, which was inhaled to 543 healthy volunteers of an age ranging from 18-25- year- old males that consisted of 1.5-2 x 10^6 cfu, followed by medical observations like regular control of body temperature haematology and x-ray imaging. These unique experimental investigation in its form was reported, that aerosol immunization with dust plague vaccine based on EV line NIIEG vaccine caused no distinct post vaccine reactions in comparison to s.c. and cutaneous immunization. The author found that s.c immunized 100 healthy males with an age of 18-25- year- old with standard live EV vaccine showed general post vaccine reaction in almost all vaccinees ranging from significant rise of body temperature above 38,5 °C to the slightest elevation and local reactions in the form of hyperemia, swelling and tenderness, sometimes accompanied by lymphadenities. Nevertheless, cutaneous immunized 5,600 healthy young males with an age of 18-25 years-old did not show any systematic post vaccination reactions. However, mild and simple reactions were observed in 2.1 %, and in 1.7 %) of vaccinees. Local inflammatory reaction in the location of inoculation and responses of regional lymphadenitis was observed in 96 % of the cases, which disappeared or significantly reduced within 7-8 days later.

The author also found that aerosol immunization with the dust plague vaccine, using the EV76 of line NIIEG vaccine strain showed prompt and high level of Immunity without no distinct post vaccine clinical reactions. however the author found that the vaccine instigated changes in the peripheral blood. It was also confirmed that the methods of immunization in field trails was quite simple in comparison to other known route of administrations of live or dead vaccines. This vaccine allows mass immunization to be carried out against plague with in a short period of time, which leads to be the best option of herd-immunization to protect a population in case of deliberate release of plague pathogen in densely populated locations or in case of epidemic outbreak of plague in endemic regions or unintended release of plague pathogen. Another experimental investigation done on monkeys also confirmed, that even low doses of aerosol

vaccine immunized twice through inhalation protected 50 % of the animals challenged in the some route with virulent strain of plague pathogen.

In contrast to killed vaccines, live attenuated EV vaccines protect quite well against aerosol challenge, but its has never been licenced and used in Western hemisphere. Furthermore there is also a scarcity of research publication that compares the live attenuated EV 76 vaccine with USP Vaccine in experimental animals or human trails (RUSSELL et al. 1995), likewise particularly EV 76 line NEIIG vaccine.

For the extent of 25 to 30 years of intensive investigations, experts in CIS concluded that, the *Y. pestis* EV 76 line NEIIG strain as the most effective prophylactic preparation against plague until now that retained its whole immunological value and stability through decades (SALTYKOVA and FAIBICH 1975) and the most intensively studied, used plague vaccine in CIS for more than 7 decades and currently, it is the only available vaccine against plague for human (FEDEROVA et al. 2007, 2008). The vaccine was found to be effective after administration by different routes including subcoutaneous (s.c), cutaneous inhalation and intramasucular (i.m) routes of injection and has been continued to be used in the countries of FSU. The life-dry plague vaccine, based on *Y.pestis* EV NIIEG line, is known with a trade name –*Vaccinum pestosum vivum siccum ad usum oralem* registeration number 93/160/21 is available and produced only in the Russian Federation (NIIEG. 2008).

Although these being the strength of this particular vaccine at the moment, there are also some concerns on live attenuated vaccines in general, that there is a fear, that the strain can reverse from avirulence to virulence, can cause a series of side effects such as reactogenicity (adverse local and systemic reactions), expression of allergic reactions, short term protection, costy, difficult in Heandling e.t.c. (tab. 2) less protection to pneumonic forms of the diseases and unable to protect from disease caused by atypical virulent strains of *Yersinia pestis*, more over, less public acceptance (WHO. 2008), that limited its global acceptance (RUSSELL et al. 1995, ALEXEEVA et al. 1997).

Live attenuated vaccines allow for infection of host cells and efficient MHC class I presentation of antigens that can stimulate strong and persistent CD8+ T cells responses, which is required for efficent protection. It mimics the natural infection and induce both arms of immune system. The central question that remains for the vaccine against disease requiring effector CD8 + T cells is whether persistent or periodic antigen (booster immunization) will be required to sustain a pool of circulating effector–memory CD8+ T cells that would be important in providing protection (BEVERLEY. 2002). Thus newly constructed live attenuated vaccines like the derivative of *Y. pestisΔlpxM*, or mutants like *Y. pestis* CO92 *yopΔ H* with their improved characteristics can be a potential candidates of vaccine which can provide a universal protection against diversified strains of plague pathogens available in different regions of the World.

Table 2. Live attenuated EV76 line NIIEG compaired to Ideal (Effective) plague vaccine

Descriptions	EV76 NIIEG	Ideal (Effective Vaccine)
Protection	Bubonic, Pneumonic, septicemic, etc. against atypical strains (?)	Must protect all forms of infection
Duration	Short term ca.1Year	Life-long protection (Ideal)
Universality	Limited	Must protect from all variants
Side effect	High (MSD 5 %)	Zero tolerance (Ideal)
№ immunization	3 booster inoculation/Year	One inoculation, induce effective immunity rapidly
Stability (Cold chain)	Stable ±, but requires cold chain	Stable no requirements of cold-chain
Safety/Handling/production	Special containment	Not needed
Cost	Expensive	Must be cheap
Public confidence	Acceptable (in Russia)	Must sustain confidence
Induced Immune system	Humoral and cellular	Humoral and cellular

EV76 NIIEG- Live vaccine *Y. pestis* EV76 line NIIEG, Data compiled from: Salytkova et al. 1975, Anisimov et al. 2004, Federova, et al. 2007, 2008, NIIEG. 2008, WHO. 2008, Beverly. 2002.

4.1.3 Plague Vaccine trails in Humans

4.1.3.1 Subunit / Recombinant Vaccines

In recent years, research efforts appear to have focused on the use of recombinant technology to design a new vaccine candidate for immunization against plague. The principal attention being focused on subunit vaccines containing recombination of two major immunogenic antigens, the Fraction 1 (F1) and Low-calcium response V antigen(LcrV).

In the United States, the subunit vaccine containing the rF1 and rLcrV as fusion protein (rF1-rLcrV) constructed in pET19b consists of the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V antigen is under development (HEATH et al. 1998, JONES et al. 2003). In UK similar proteins but equal concentration of both components (rF1 + rLcrV) is under development. Both vaccines provided efficient protection against bubonic and pneumonic plague in murine animal models against $F1^-$ and F^+ strains of *Y. pestis* and it was also reported to be well tolerable in Human trials phase 1 (TITBALL and WILLIAMSON 2001, 2004, ANDERSON. 1996, WILLIMSAON. 1999, WILLIASON et al. 2001, 2005).
Better protection was observed when it was given as a subunit/ fusion protein (rF1-rLcrV) to the mice as either F1 or V alone against s.c. challenged with $F1^+$ *Y. pestis* CO92 strain (HEATH et al. 1998). Better protection by antibody directed against both, F1 and V antigens might occur by counteracting both the antiphagocytic activity associated with F1 (BURROWS. 1963) and the virulence enhancing activity associated with secreted V antigen (NAKAJIMA et al. 1995).

Similar results were also achieved when it was given as recombinant/ equi-molar concentration of both antigens (rF1+ rLcrV) that provided a protection of BALB/C mice against a subsequent s.c. challenged of 10^6 LD_{50} *Y. pestis* strain GB, while in contrast control mice challenged with similar doses of *Y. pestis* developed disease and animals died at day 5 post infection (GRIFFIN et al 2005). The predominant IgG subclass raised to both F1 and V antigen was IG g1, that correlates with protection against plague in the mouse model was reported (WILLIAMSON et al. 1999). But guinea pigs immunized with (rF1+LcrV) vaccine on the contrary were protective only against low dose of of *Y. pestis* GB strain. As the doses increased there was a loss of protection and only 50 % of the animals survived, where all mice were protected against s.c. challenge with the same doses. All of the immunized guinea pigs were seen to have buboes including the lowest challenged group and in almost all cases, under the right forelimbs. Bacteriological analysis confirmed *Y. pestis* isolates in the liver, spleen and lungs. More significantly, the levels of Ig1 class antibodies were notably variable against both proteins (JONES et al. 2003).

In concerns to guinea pigs, according to the Russian national standard of plague vaccine resolution steadfast, that the potential efficacy of plague vaccine strain requires that at least 50% of guinea pigs should be protected against s.c. challenge with 200 MLD (2000 cfu) of virulent strain

(ANISIMOV et al. 2002) and guinea pigs belongs to one of the multispecies notably included in Plague research in CIS (ANISIMOV. 1999, 2004, ANISIMOV et al. 2002, FEODOROVA. 2007, 2008).

In addition to the two major imunogenic antigens of *Y. pestis,* studied in depth, there are also a number of proteins known as Yops and Pla in eliciting antibodies and some of them provides partial protection (YoD) from s.c infection in mice model (ANDREWS et al. 1999). Recent studies made by LI et al. (2008), showed that 10 other novel antigens that provided an experimental evidences for immunogenicity in using an antigen microarry containing more than 140 *Y.pestis* virulent associated proteins in detecting antibody responses in plague patients.

Although LcrV protein belongs to one of the major immunogenic protein, it was also reported that LcrV triggers the release of interleukin-10 (IL-10) by host immune cells and supresses proinflammatory cytokines such as TNFα and TNFγ as well as innate defense mechanisms required to combat the pathogenesis of plague which may preclude the use of LcrV as a human vaccine (NAKAJIMA. 1993). *Y. pestis* can bypass protective antibodies to LcrV and activation with IFN-gamma to survive and induce apoptosis in murine macrophages (NOEL et al. 2009). The three human pathogenic Yersinia species all harbors a 70 kb virulence plasmid LcrV (FILIPOVA. 1990, BURBAKER. 1991, CHERPANOV. 1991). DNA sequencing of the LcrV antigen revealed that two evolutionarily distinct types of V antigen exist in *Yersinia* spp. One type is expressed by *Y. enterocolitica* serotype O:8 (designated LcrV-YenO8 or V-O:8), and the other type is expressed by *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serotypes O:3, O:9, and O:5,27 (LcrV-Yps or V-O:3). More recently, it has been suggested that *Y. pestis* strains possess their own V-antigen type, V-Yp (ANISIMOV et al. 2004). The Antisera from two LcrV variants did not show cross-protection. Protection was observed in mice if the immunizing LcrV antigen was from the same type as LcrV antigen by the infective strains (ROGGENKAMPF et al. 1997). It appears that they are at least , potentially interchangeable LcrV antigen variants and it is possible that in the diverse population of *Y. pestis* circulating in Asia there are more variants (ANISIMOV et al. 2004). Thus this peculiarity of only self recognizing characteristics of LcrV might deter in active immunization and in providing universal protection against highly interchangeable variants which might be found in the diverse dynamic populations of *Y. pestis* circulating in natural foci found around the world.

Individually, the F1 and V recombinant proteins are immunogenic and protective against challenges. In combination, they provide protection against very high challenge levels with virulent plague in small animal models. The predominant animal model used in most of the research activities, achievement were/ are based on mice (WILLIAMASON et al. 1997, 2001), where guinea pigs are categorized as bad model to study plague pathogenesis, immunology and vaccine developments in USA and UK (WORSHAM. 2004). It is also known that mice and guinea pigs respond differently to the plague pathogen and that LD_{50} of 10 cfu of the poorest virulent strain of *Y. pestis* can kill guinea pigs, thus while research results depend on the animal used, it make difficult to translate from different animal models to human use. Translation of experimental

results of vaccine based on single animal species model might rise a questions of acceptability, where it is known that a number of rodents are infected and are sensitive to the plague microbe.

In Russian the major focus of plague vaccine research approaches concentrates on the development and investigation of live plague vaccine as improved or new vaccine (FEDEROVA et al. 2007). Currently a dozen of strains of *Y. pestis* which fulfils the Russian national standards of major requirements of qualification for new or improved vaccine development compared to the "Etalon" reference vaccine strain of live EV 76 line NIIEG (ANISIMOV et al. 2002, FEODOROVA et al. 2007, 2008). One of the current candidate, the *Y. pestisΔlpxM*, has shown, as new improved vaccine with better efficacy, more protection and less toxicity in all three animal models in comparison to the live EV76 line NIIEG against infection induced with virulent wild-type 231 strain of *Yersinia pestis* (FEDEROVA et al, 2007,2008).

In contrast to the above analysed vaccine candidates in USA and UK, the Russian national criteria for plague vaccine-testing and candidate strains for new or improved plague vaccine developments is currently applicable for the development of new or improved vaccine against plague pathogen in Russia (ANISIMOV et al, 2002, FEODEROVA, 2007, 2008). In contrast to the west, the live attenuated vaccine of EV 76 NIIEG line is the most thoroughly studied plague vaccine in FSU/ CIS since 1936. It was constantly proofed to be the best effective, stable prophylactic preparation against plague, with its constant cultural morphological-biochemical characteristics and immunogenicity that was proved through an investigation done on the strain after 30 years of storage. Although the EV 76 strains used as a vaccine showed various results depending on the laboratories (RUSSEL et al.1995), EV76 NIIEG line was proofed to be stable. All important immunological, biochemical and morphological characteristics analysed were consistent, showed no signs of alteration was seen after 3 decades of storage (SALYTKOVA et al. 2000, ISUPOV and LEDVANOV 2004). It is also important to note that each year, over 100,000 CIS citizens get anti-plague vaccine based on the Live EV 76 NIIEG line, mostly geologists, cattle breeders, and live-stock expertise (Veterinarian, Animal nutritionist, Zoologists, e.t.c.) most likely to be in contact with plague-infected animals. The vaccination is valid for at least 6 months (DOMARADISIJ and ORENT 2006, FEODEROV. 2007, 2008, ISTC. 2008).

In most of the cases protective efficiency of new vaccine candidates researchers, developments and success in UK or USA are mostly compared F1+V or F/V fusion vaccines with killed USP vaccine. There is no comparative study made between the live attenuated EV strain of NEIIG line and F1/V or rF + rV yet. Thus, it was reported that, the benefit of a vaccine containing both F1 and V is, that F1 should protect against variant strains of *Y. pestis*, which might be altered in the amount, or composition of V antigen, in a manner analogous to that by which V antigen protects against F^- strains. As another potential advantage reported for an F1/V multi-component vaccine would be to protect individuals who may be non-responders to one component of a vaccine.
A number of studies have clearly demonstrated that flagellin is a potent adjuvant that promotes robust immune responses when it is given with a protein antigen. MIZEL et al. (2009) evaluated the efficacy of a recombinant protein vaccine composed of a single fusion protein containing flagellin

and two protective antigens of *Yersinia pestis* (F1 and V) in eliciting protection against respiratory challenge with *Y. pestis*. It was reported that, the fusion protein (Flagellin-F1-V) retained full Toll-like receptor 5-stimulating activity in vitro. Using a prime-boost immunization protocol, they found that flagellin-F1-V elicits robust antigen-specific humoral immunity in mice and two species of nonhuman primates an immune mice were fully protected against intranasal challenge with 150 mean tolerated doses of *Y. pestis* CO92. In immune mice, the bacteria were completely cleared within 3 days after challenge. Flagellin-F1-V exhibited full stability for at least 297 days at 4°C and at least 168 days at 25°C. At between 29 and 84 days at 37°C, the protein exhibited a loss of biological activity that appeared to be associated with a substantial change in protein diameter, possibly due to oligomerization.

Not less important, is also, that a number of atypical strains, with intraspecific diversity of *Y. pestis* isolated from different natural foci, which are not yet known in Western hemisphere, but persist on all continent save Australia, has never been included in sort of vaccine studies yet. Most of the research results in west are based on strains which are genetically identical to over 98 % and stored in the laboratories for long period of time, like strains of *Y. pestis* CO92, *Y. pestis* KIM, *Y. pestis* GB, unlikely which differ with the strains found in natural foci known in CIS and other regions of the world, that narrows the shield of protection of vaccine based recombinant of F1 and V antigens (PARKHILL et al. 2001, DENG et al. 2002). Plague microbe population inhabiting in varies ecosystems are dynamic and responsible for sporadic out breaks which are consistently reported to the WHO. Thus, it is essentially needed to gain full knowledge of them in order to develop an effective, stable, efficient, tolerable, prophylactic measures against plague for global use and has a universal protection and acceptability and the subunit/recombinant vaccine must be proofed for fulfilling these requirements.

4.1.3.2 Non-cellular (Synthetic) Vaccines

Techniques have been developed for locating and defining epitopes on bacterial or viral proteins and the possibility to synthesize peptides chemically corresponding to these antigenic determinants. They are designed to overcome the drawbacks of classical vaccines like, safety, difficulties in processing, reversibility, genetical variations of microbes within themselves, multiplicity of the microbes and more over post-immunization side effects, like toxicity and stability (ARNON. 1997, HARARI et al. 1997, MULLER. 1982).
In the beginning of the 70th PITROV et al. [quoted by ALEXEEVA. 1997] showed for the first time, that series of synthetic polyelectrolyte's, substances that does not have natural analogue, were able to express an immunomodulating effects. This observation was based on the facts that immunization with the given polymer complex enhanced specific types of immunogenicity, that were able to substitute the function of helper T (T_H) cells. This experimental evidence may give a new opportunity to produce vaccines based on non-immunogenic or weak immunogenic substances. It might overcome the problems of phenotypical corrections of genetically controlled immune responses and might provide protection to all vaccine contingents independent of the

genotype of the immunized subject. Experimental investigation of the conjugates obtained from capsule antigen designated as F1 and F1A, based on the methods of extraction was reported that, mice immunized with the combination of polymers, monomers or tetramers of non immunogenic polysacchride (PS) and synthetic polyelectrolytes of cationic nature called "sopolymer 4-vinylpyridin and 4-vinil-N-ethylpyridinbromid" or (ME-3) complex enhanced the survival rate and immune responses complex against plague infection (ALEXEEVA et al. 1997). The combined vaccine was designated as Chemical plague vaccine (CPV). It was also reported that significant improvement in expressions of 1st and 2nd cellular immunity responses to F1A, and prolonged 2ry humoral responses was observed (ALEXEEVA et al. 1997). That indicates an alternative option of constructing a vaccine against plague based on capsule antigen and polyssacharides with les or non immunogenic substances.

DALVADYANTS et al. (1997) studied the experimental model of CPV (F1A-PS-ME3) described, as a soluble substance that stored its immunogenicity for a longer period, significantly less toxic and less allergic in comparison to live attenuated EV NIIEG line vaccine or killed whole cell vaccine (USP). The observation was based on experimental studies made on animals and human volunteers. It also showed an efficient protection against virulent strain of *Y. pestis*, when it was used as *revaccination* (booster-vaccine) than as primary vaccination. Thus, the vaccine was described to be even superior to live attenuated EV NIIEG vaccine in specific situations, in that it was more stable and can stores its specificity for 15 years and it can be used with external prophylactic substances like antibiotics or immuno-modulators. His experimental studies on laboratory animals (mice, guinea pigs, primates) and clinical investigations of volunteers done lead to suggest, that the CPV has such a good quality to be implemented as a revaccination (*effective booster quality*) with minimal clinical adverse post-vaccine reactions was reported and it was also reported that it reactivates the immune system better than live plague vaccine (LPV) and USP vaccine. Specific antibody titers was observed in a range of (1:2-1:32) to the F1 antigen in 36 % of volunteers inoculated in the transition period to revaccination. 30 days after booster immunization the titer rose to 96 % to F1 antigen, ranging from 1:64-1:4096 in 54.13 % of the case. Whereas, revaccination with LPV increased the titers in only 10 % of the volunteers, with maximum antibody titers less than 1:64 in 20 % of the participants. Indirect analytical methods used to indicate the efficiency of revaccination of volunteers with live and chemical plague vaccines showed specific immune stimulating substances of the CPV which were preserved for 3 to 6 months. In 84 % of the volunteers, specific antibodies to F1 were detected during the period with titers ranging 1:6-1:512 in 27.4 % cases.

Recent report what is known about the CPV was an investigation made by DALVADYANTS et al. (2005). He used the biocomponent of capsule antigen (F1) and OSA from Russian word (**O**snovnoi **S**omatichiskii **A**ntigen [main somatic antigen]), i.e. an antigen isolated from experimental animals like white mice, guinea pigs, hamadryas pavian, and from human in clinical trials for immunization. The study consisted of 100 healthy human volunteers, with an age of 18-30 years old, gender not specified. Volunteers were immunized s.c. with live plague vaccine serie 66, № 559; 3x 10^8 cfu initially and were randomly selected and grouped in to three. Group 1 consisted of 52 individuals,

group 2 consisted of 16 and group 3 consisted of 14. Group 1 were revaccinated s.c. with CPV of 12 µg concentration in a volume of 0,5 ml in 0.9 % saline solution /head. The second group were revaccinated with LVP vaccine serie 66 with the some dose as the primary immunization per-head and group 3 were placebo (control) groups. This study compared the efficiency of CPV to LPV and included control group (placebo). It investigated the frequency of general reaction of the volunteers revaccinated with CPV or LPV like, body temperature, depression, general feelings, headache, vomiting, weakness. Significant differences of physiological parameters between CPV and LPV vaccineetes were not observed. Further, the indicators of local reaction in hrs like pain, hyperemia, oedema were also examined. Pain were observed in 90 %, 93% of the CPV and LPV vaccineets respectively in the first 24 hrs. Further observations showed that after 72 hrs 11 %, 25 % of CPV and LPV Vaccinates respectively feel pain. Whole blood and serological investigation was also done in order to elucidate the dynamicity of antibodies against the CPV and F1 antigen and for the exclusion of toxicity of the vaccine. It was shown that CPV was less reactogenic, while protein products produced under stress conditions were not detected. CPV is easy of handling, well acceptable. Serum antibody measured 6 months after revaccination were detected in 85 % of the vaccinates inoculated with CPV used as booster vaccine and in 64 %, of individuals injected with LPV used as revaccination, that indicates CPV is more active and efficient in inducing antibodies. In addition serum antibody measured against F1 antigen, 30 days post booster injection with CPV and LVP were detected in 94 %, 47 % of the cases respectively. Thus, the Authors proposes the importance of including the CPV vaccine as booster vaccine in cases of combating against plague outbreak or in plague control and prevention. No experimental studies are available on CPV as “standalone” which a vaccine against plague is supposed to be.

Despite the fact that synthetic vaccines may have several advantages in comparison and have been shown to elicit neutralizing antibodies in certain viruses of animal pathogens, like Foot- and mouth disease, rabies virus, there are also a number of disadvantages, which needs consideration and further investigations. Synthetic peptide vaccines are poorly immunogenic, needs adjuvants, they are linear, lack any tertiary or quaternary conformation while epitopes are not composed of linear arrays, of contiguous aminoacids, but rather three-dimensional shapes in its native form. Most B cells epitopes are nonlinear (discontinuous) and therefore not reproduced by peptides. Short linear peptides can be recognized by T-Cells, when bound to MHC protein. May be to narrowly specific, not protecting against natural variants. Single-epitope vaccine may readily select mutants in the field (HARARI. 1997). In order to produce vaccines based on synthetic vaccine these merits must get solutions. There is no licenced purely synthetic vaccine for human use yet.

4.1.4 Plague Vaccine studies in Animal Models

4.1.4.1 Live Attenuated Vaccines

Recently new improved vaccine like, the *Y. pestis* EV NIIEG derivatives with deletion-insertion in *lpxM* gene (hexa-acylated-lipid A) are under experimental levelin animal models . The *lpxM* mutants vaccine construction was reported to be, less reactogenic in comparison to its parental strain and provided better protection in three species of experimental animals against s.c. challenged virulent strain of *Y. pestis* 231 (FEODOROVA et al. 2007). It was also reported that, no bacterial cells were detected from organ speciemen cultured from animals' immunized with *Y. pestisΔlpxM* vaccine. That suggests, there are no signs of residual virulence related to the new improved vaccine.

Comparative studies done by FEODOROVA et al. (2007), in regards to the efficacy of protection between *Y. pestisΔlpxM*, the new improved vaccine and the parental EV76 NIIEG line vaccine in all three animal models tested showed that *Y. pestisΔlpxM* provided a significant level of protection against challenge doses of 1.2 x10^4 to 2 x10^4 cfu (LD_{50} = 1,2-2,0 x 10^3 cfu) of wild-type 231 strain. In contrast, immunized animals with parental vaccine were not protected was reported. Also a protection of 57-86 % was provided in outbred mice immunized with *Y. pestisΔlpxM* vaccine depending on the concentration of doses of immunization. It was observed that immunzation doses with higher concentration of bacterial cell significantly showed more protection as low concentration. For instance a vaccine with 10^9 cfu of *Y. pestisΔlpxM* was capable of protecting 85,7%, where as vaccine with a concentration doses of 10^5 cfu protected only 14 % of the outbred mice and no differences were seen with regard to the parental vaccine, that both high or lower concentration did not provide any protection.

Furthermore, it was reported that, the *Y. pestisΔlpxM* elicited modest protection (no more than 40% of survivals) in Balb/C mice whereas, *Y. pestis* EV NIIEG do not show any protection; all immunized animals did not survive. It was reported that, among guinea pigs, a marked protective efficacy against challenge with 1,2 x10^4 cfu of wild–type was achieved following a single dose of the *Y. pestisΔlpxM* strain between 10^7 and 1,5 x10^{10} cfu/ animal. No protection of guinea pigs was seen following immunization with the *Y. pestis* NEIIG regardless of the level of concentration even after higer doses of 10^{10} cfu immunization (FEODOROVA et al. 2007).

In similar studies it was reported, that highly attenuated phenotype of *Y. pestis* CO92 *yopΔ H* (YopH- a protein tyrosine phosphatase mutant), provided higher protection against s.c. or intranasal (i.n.) challenged out bred CD1 mice that corresponds to bubonic and pneumonic models of plague respectively (BUBECK and DUBE, 2007). Further observation was made, that vaccination with live attenuated *Y. pestis* CO92 *yopΔ H*, i.n. with dose of 10^7 cfu provided 100 % protection

against the some route of challenge with ~10^5 cfu of *Y. pestis* CO92 virulent strain, and s.c. immunization showed 70 % of protection against i.n. infection. Interestingly it was found that mice vaccinated i.n was 100 % protected against s.c. challenges with virulent strain of *Y. pestis* CO92 (BUBECK and DUBE 2007).

Thus, the current challenge is to develop safe, universally effective vaccine against the deadly form of plague, the pneumonic plague, and the most likely way of disease transmission in bioterrorism. Toady, it is a constant observation that vaccines targeting intracellular pathogens like *Yersina* must induce both arms of immune system. Although immunity towards intracellular pathogens is often depend upon the generation of $CD8^+$ memory T cells, for long-lasting and effective protection, the humoral part of immune system plays its main role in the effective activation of the antigen-specific immune response, in antigen-uptake, and antigen presentation (ISUPOV and LEDVANOV 2002, FEODOROVA et al. 2007, SMILEY. 2008). Thus, any forms of plague vaccine development must be able to fulfil the drawbacks known by killed vaccines.

Live attenuated vaccines allow for infection of host cells and efficient MHC class I presentation of antigens that can stimulate strong and persistent CD8+ T cells responses, which is required for efficient protection. It mimics the natural infection and induce both arms of immune system. The central question that remains for the vaccine against disease requiring effector CD8 + T cells is whether persistent or periodic antigen (booster immunization) will be required to sustain a pool of circulating effector–memory CD8+ T cells that would be important in providing protection (BEVERLEY. 2002). Thus newly constructed live attenuated vaccines like the derivative of *Y. pestisΔlpxM*, or mutants like *Y. pestis* CO92 *yopΔ H* with their improved characteristics can be potential candidates of vaccine which can provide a universal protection against diversified strains of plague pathogens available in different regions of the World.

4.1.4.2 Heterologous Vaccines

A vaccine that confers protective immunity against a pathogen that shares cross-reacting antigens with the microorganisms in the vaccine and provides cross-protection is hetrologous vaccine. Ideal vaccine must be able to protection from both infectious agents simultaneously by utilizing a single vaccine so to say “kill two birds with one stone”. Studies on microorganisms of the same family and genera were given priorities based on their cultural, morphological, biochemical and common antigens, if they are capable of protecting from one or both agents (EBLÉ et al. 2006).

Recent observation on the genome plasticity in *Y. pestis* led to the proposal that *Y. pestis* is a recent clone of *Y. pseudotuberculosis* that arose between 1,500 and 20,000 years ago and more than 95% of genetic identity (ACHTMAN et al. 1999). In spite of what they have in common, the causative agent of pseudotuberculosis lacks F1 and Murine toxin, which plays an important role in inducing immunity and toxicity in *Y. pestis*.

One of the first trials of immunization of animals against the plague microbe with *Yersinia pseudotuberculosis* was carried out by ZLATOGOROFF (1904, 1918) [cit. by DOMARADISKIJ. 1998], unfortunately there was no positive result. In contrast, MACCONKEY (1908) and ROWLAND (1912) [cit. BY DOMARADISKIJ. 1998] inoculated animals with live and killed cell cultures and achieved a clear expression of immunity against plague. BOY (1932, 1933) [cit. by POLLITZER. 1954] used a formalin killed *Y. pseudotuberculosis* cells in experimental trails as a vaccination for human in Madagascar against plague was not unsuccessful. In further studies THAL, (1955, 1956) [cited by DOMARADISKIJ. 1998] showed that a single inoculation of guinea pigs with live*Y. pseudotuberculosis* serotype IV boullion (avirulent strain 32) induced a stable immunity. Another similar study reported by SIMONET et al. (1985), indicates that specific immune response to *Y. pestis* was induced when mice were immunized with viable bacteria from a virulence plasmid cured strain of *Y. pseudotuberculosis*. Also, this antigenic stimulation generated specific protection against virulence-plasmid-harboring *Y. pseudotuberculosis*. Recently it was reported that two oral immunization of live attenuated vaccine based on *Y. pseudotuberculosis* IP32680 provided a protection of 88 % of mice against subcutaneous challenged *Y. pestis* CO92 (BLISNICK et al. 2008). In spite of the capability of *Y. pseudotuberculosis* in inducing immunity against bubonic plague, several questions remain unanswered. For example, the question concerning the mechanism of immunity against plague as a result of an inoculation of *Y. pseudotuberculosis* or, which antigens of this microbe are necessary for the development of immunity or the level of protection? If it does protect against pneumonic plague, the most feasible route of infection in case of intentional spread of the pathogen? Or plague caused by a number of atypical strains available in nature around the world and are know to be virulent for human?

Immunization with *Y. pseudotuberculosis* carrying the recombinant plasmid signified as KM100 containing, the major protective antigen of the *Y. pestis*, capsule antigen (F1) and attenuated auxotrophic strain of *Y. pseudotuberculosis* KM99 were reported to show some low level of protective in mice and guinea pigs challenged s.c. with virulent strains of *Y. pestis* and *Y. pseudotuberculosis* (DARMOV et al. (1997).

Studies made by TAYLOR et al. (2005) showed that oral immunization of BALB/C mice with a *dam* (gene encoding DNA adenine methylase) mutant of *Y. pseudotuberculosis* strain IP32953, protected against a s.c. challenge with 100 MLDs of *Y. pestis* strain GB and an intravenous challenge with 300 MLDs of *Y. pseudotuberculosis* IP32953. It was also reported that no bacteria were detected in spleen homogenates in both cases, where as naive mice challenged s.c. with 100 cfu. *Y. pestis* of GB died within 10 days. Although mice immunized either by oral or i.v route showed protection against *Yersinia pestis* challenge, immunization with Dam-deficet *Y. pseudotuberculosis* offered the highest levels of protection with complete protection seen if the dose of 10^6 cfu or greater was reported. That indicate the possibility of developing a bivalent vaccine.

Thus, uses of pathogens that naturally infect mucosal surfaces are of interest as vaccine vectors for the delivery of foreign antigens to the immune system to stimulate both systemic and mucosal immune responses, which may protect against bubonic and pneumonic plague. They can also be

administered orally. Therefore, *Salmonella* based vaccines are attractive as carriers of protective antigen like F1, V-antigen YOP e.t.c. Intragastrically and intravenously application of the recombinant vaccine that consisted of an attenuated *S. typhimurium aroA*$^-$ strain, which expressed the F1, capsular antigen of *Y. pestis* protected mice against the virulent strain *Y. pestis* GB (OYSTON et al. 1995, TITBALL et al. 1997). It was also reported that the protection was correlated with the induction of high titers of immunoglobulin G in serum samples and T-cell response Attenuated *S. enterica* serovar typhimurium SL3261 *(aroA*$^-$) expressing the *Y. pestis* V-antigen immunization was shown a protection (30 %), when mice were challenged s.c. with 97 cfu of *Y. pestis* GB (GARMORY et al. 2003). V-antigen specific IgG was detected in the serum of all mice. However, the concentration of specific antibody induced in mice which survived the plague challenge were not significantly different to some antibody concentrations induced in dead animals.

Similar studies done by ANDREWS et al. (1996) also showed that, Fraction 1 capsular Antigen (F1) purified from *Y. pestis* CO92 and from an *E. coli* HB101 /F1 recombinant strain protected 70 to 100 % of the mice challenged s.c. with 100 LD_{50} of wild-type of *Y. pestis* CO92 where as the licenced killed-whole-cell (USP) plague vaccine protected 50 % of the mice. After aerosol challenges with the same doses 65 to 84 % of the mice immunized with recombinant vaccine were survived, where as the plague vaccine protected only 1 mouse of 9 mice.

Oral immunization with attenuated *S. enterica* serovar Typhimurium vaccine strains in mice has generally constituted a model for oral immunization with attenuated *S. enterica* serovar Typhi vaccines in humans, since *S. enterica* serovar Typhi is not known to be virulent in any animal model by the oral route of infection. A recombinant strain of attenuated *S. enterica* serovar Typhi surface-expressing *Y. pestis* F1 generated by transforming strain BRD1116 (*aroA*$^-$ *aroC*$^-$ *htrA*$^-$) (LOWE et al. 1999) with plasmid pAH34L encoding the *Y. pestis caf* operon, protected 65 % of intranasally immunized mice, (twice with a dose of 1X 10^8 cfu) 13 out of 20 from lethal challenges with *Y. pestis* (GRIFFIN et al. 2005). Immunogenic antigens of *Y. pestis* like F1 or V encoded in closely related species of *Y. pseudotuberculosis* strains or other enteropathogenic species like, Salmonella and Escherichia showed that there is a possibility of developing a bi-or multi-valent vaccine, but that needs further investigation. Multivalent vaccines can have great disadvantage such as, the increasing complexity of antigen mixture may make of vaccine associated adverse events and may also have advantage like, they will induce immune responses different from those engendered by single antigens or single organism products.
In addition various forms of vaccine trials, such vaccines based on non-cellular extracts (synthetic/chemical), vaccines based on recombinant antigens of other closely or distantly related bacteria, i.e. *Y. peudotubreculosis* (DARMOV et al. 1997), *S. typhmurium* (OYSTON et al. 1995; TITBALL et al. 1997) *E. coli* (ANASIMOV. 1999) were also studied. Some of these vaccines demonstrated some level of protection, in experimental animals and few human volunteers in specific situations.

ANISIMOV'S. (1999) investigations, including 42 strains of *Y. pestis*, 1 strain of *Y. enetercolitica*, 41 strains of *E. coli*, in which the major capsule antigen (F1) gene were introduced by plasmids

showed that, a recombinant capsule antigen (F1) of *Y. pestis* expressed by *E. coli* expressed high protection against the infection of virulent "wild" strains of *Y. pestis* to guinea pigs and mice for the first time. Serological studies of recombinant antigen of F1 based on 42 strains of *Yersina*, induced 1,000-10,000 times higher antibody titers than the reference vaccine strain of *Y. pestis* EV NIIEG line. Recombinant antigen based on 10 strains of *Salmonella* spp. showed, that recombination based on *S. minnesota* protected the animals to the same level of the live EV 76 vaccine of NIIEG line. Most of what is known about recombinant vaccines based on other organisms are targeted to s.c. infection, and less is known about aerosol or atypical caused plague infection.

4.1.4.3 DNA Vaccines

The first report to the successful expression of naked plasmid DNA in mouse muscle tissue was by WOLFF et al. (1990). Three years later the potential for the development of new vaccines against infectious organisms was demonstrated when DNA encoding the nucleoprotein of influenza virus, pseudorabies virus were shown to induce antibody, specific T-cell responses, and protective immunity against viral challenge (ULMER et al. 1993, CHANG. 1998). Since then, DNA vaccine has been prepared and tested against a wide range of infectious disease initially against infections caused by viral diseases (WANGE et al. 1998, BOYLE et al. 1999).

The DNA vaccines are the simple rings of DNA containing a gene encoding an antigen and a promotor/terminator to make the genes express in mammalian cells. They are promising new approaches for generating all types of desired immunity: cytolytic T lymphocytes (CTL), T helper cell antibodies (SMILEY. 2008), Whilst being a technology that has a potential for global usage in terms of manufacturing ease, broad population administration and safety (FYNANA, et al. 1993). There are few studies reported on the use of DNA vaccine against bacterial infections, although the potential has been demonstrated for *Mycobacterium tuberculosis* (HEWINSON. 2005) *Clostridium tetani*, *Corynebacterium pseudotuberculosis* in sheep (CHAPLIN et al. 1999), *Chlamydia psittaci* (VANROMPAY et al. 1999), *Bacillus anthracis* (STEPANOV et al. 1996) and some intracelluar infections.
One of the impediments to successful vaccination against the aforementioned infectious agents like plague, glanders and melioidosis, are likely they require a cellular as well as humoral immune response for protection (ROBERT et al. 2000, MICHELLE et al. 2005). In this regard, although killed vaccine like USP in plague is efficient at inducing antibody responses which alone is not sufficient to provide complete protection, only vaccines based on live attenuated organisms be able to induce cellular immunity efficiently, which is crucial for intracellualr pathogens like *Yersian pestis*. It should be noted, however, that wide spread use of life attenuated vaccine might be precluded by practical constraints such as manufacturing, post-immunization adverse reaction, safety concerns or short term protection and needs of multiple booster immunizations. Thus, it was demonstration over the last decade that plasmid DNA vaccines can induce both humoral and cellular immune responses in a variety of murine and primate disease models and might overcomes the drawbacks

of killed or attenuated vaccines known yet. This has engendered considerable excitement in the vaccine community.

The historical basis for DNA vaccines rests on the observation that direct *in vitro* and *in vivo* gene transfer of recombinant DNA by a variety of techniques resulted in the expression of proteins that is able to elicit protective immunity (ULMER et al. 1993). DNA vaccines provide prolonged antigen expression leading to amplification of the immune response and appear to offer certain advantages such as ease of construction, low costs of mass production, high levels of temperature stability and the ability to elicit both humoral and cell-mediated immune response (GURUNATHAN et al. 2000).

The endogenous expression of antigen from DNA introduced into host cells lead to peptide presentation with the major histocompatibility complex class I (MHC-I) which is ideal for induction of cytotoxic T-cell response. Therefore, DNA vaccines have been primarily considered for use against intracellular pathogens such as viruses (GREGERSEN 2001, SAIKH et al. 1998). Nevertheless, the observe ability of DNA vaccines to elicit both cell-mediate and humoral immune responses paved the way for their assessment as expressers of soluble, secreted bacterial antigens conferring immunity presumably by eliciting the classical MHC-II mediate humoral response (FELTQUATE et al. 1997). It was also known that the efficacy of such DNA vaccines was found to vary from case to case and depends on the nature of the individual antigen, on the vaccination mode and on the subcellular location in which the antigen was expressed (BOYLE et al. 1997, TORRES et al. 1999). It has been also suggested that the use of DNA vaccine may be complicated by fundamental differences between prokaryotes and eukaryotic genes (e. g. codon usage, and gene products due to different cellular machinery), leading to the poor expression of a proteins correctly folded.

It was reported by HAIM et al. (2003), that DNA vaccine against plague, based on plasmids expressing derivatives of *Y. pestis* capsular antigen F1, devoid of its putative signal peptide (deF1) induced an effective antibody response and conferred protection against high dose of infective virulent *Y. pestis* strain of Kimberley53 (HAIM et al. 2003) and intramuscular (im) and intra-dermal (id) immunization of different mice strains, out-bred and inbred mice challenged with 50 LD_{50} s of *Y. pestis* 3 weeks post immunization provided practically fully (98,4 %) protection, independent of the mouse strain or the mode of vaccination and the GMTs (geometric mean titer) of specific anti-F1 antibodies in this various groups ranged from 4,000 to 19,000.

Another study reported by WILLIMASON et al. (2002) demonstrated, that co-immunization with plasmid DNA cocktails primes mice against anthrax and plague. It was demonstrated, that the protective antigen *PA* of *B. anthracis* and V antigen of *Y. pestis* are potent immunogens and candidate vaccine subunits. Mice primed with a combination of each plasmid and boosted with a combination of the recombinant proteins were fully protected against challenges with *Yersina pestis* whereas mice immunized with plasmid DNA encoding V antigen and boosted with rV were only partially protected (50%) against the same challenge dose. This indicates that, DNA immunization can be an effective strategy against plague as recombinant proteins, both PA and V

antigen, are potent immunogens inducing high titres of circulating IgG in the mouse model. Attempts to understand the advantage in combining plasmid DNA constructs encoding, rPA and rV respectively into a single immunization and its efficacy to protect from single or both infectious disease needs further investigations.

The possibility of combing a number of plasmids in a cocktail to create a multivalent vaccine could be very attractive as well as conceptual and logistical advantages can be expected. There could be practical advantages in that, that one plasmid may act as an adjuvant for a co-administered plasmid. WANG et al. (2004) studies, demonstrated that a novel DNA vaccine expressing a modified V antigen (LcrV) of *Y. pestis*, with a human tissue plasminologen activator (tPA) signal elicited strong V specific antibody responses and the tPA/V DNA vaccine protected mice from intranasal challenge with lethal doses of *Y. pestis* (KIM strain).

DNA vaccination might provide several important advantages over current vaccines: 1) DNA vaccines mimic the effects of live attenuated vaccines in their ability to induce major histocompatibility complex (MHC) class I-restricted CD^+ T-Cell responses, which may be advantageous compared with conventional protein-based vaccines, 2) DNA vaccines can be manufactured in a relatively cost-effective manner and stored with relatively ease, eliminating the need for a "cold chain" (the series of refrigerators) required to maintain the stability of vaccines during distribution, 3) Induces both, humoral and cellular immune responses which are necessary to protect from intracellular pathogens like *Y. pesti,* 4) Unable to reverse to virulent.
These being the advantages of DNA vaccine theoretically, there are also some area of concerns such as, possible integration into chromosomal DNA, leading to insertional mutagenesis or oncogenicity, the possibility of the vector plasmid in transforming the microflora of the host to antibiotic resistance and possibility of germline transmission, induction of autoimmune disease including anti DNA-Antibodies (MURPHY. et al. 1999, GLENTING and WESSELS 2005). Worldwide, there are no licenced vaccines based on DNA against bacterial infection yet.

Currently, there are a number of novel approaches to design a vaccine, molecular-biotechnical methods like, identifying essential immongenic proteins as well as virulence factors of the pathogen, single or recombined to develop a vaccine (WILLIAMSON. 2001), sequencing different strains of Yersina or Burkholderia species to understand their relationships, the role which each or groups of genes do paly (PARKHILL et al. 2001, DENG et al. 2002, CHAIN et al. 2006, GARCIA et al. 2007). Furthermore, the availability of cross checking technologies of purity, stability, safety, durability e.t.c., of vaccine components (WHO. 2007, CORNEILS. 1989, 2002, GRANDI. 2001), or deleting the immune suppressive and reactogenic encoding genes (FOEODROVA et al. 2007,2008, BUBECK and DUBE 2007, PALMER et al. 1999).

The advances in our understanding of the basis of immunology of the host on one hand and the host-pathogen interaction on the other hand facilitates the development of new vaccines which are based on a rational choice of pathogen-derived immunogenic sequences in order to induce effective, long-term protection with less, ideal with no toxicity (ROSENTHAL and ZIMMERMANN

2006, FEODEROVA et al. 2007, 2008), that will all lead us to the new era of vaccine production against pathogen that causes plague, glanders and melioidosis, which are relevant to public health and security concerns.

The ease of manipulation of gene sequences, isolation of the target gene, cloning, expression and investigation of the role of specific protein *in vivo* and *in vitro* and novel delivery systems are emerging which can activate specific pathway of immunity. New vaccine developments directed against intracellular microorganisms like *Yersinia pestis* must induce both arms of immunsystem, the humoral and cellular immune responses must be provoked. The Vaccine must be able to activate especially cytotoxic T-cell responses, which have been difficult to induce with some other forms of vaccines like killed vaccines. New or improved vaccines must provide universal protection against virulent atypical strains which are available worldwide (ISUPOV and LEDVANOV 2002, HEALEY et al. 2005, KALACHEV et al. 1997, KETHEESAN et al. 2001).

4.1.4.4 B Antigen and BaSoAn

B antigen and **Ba**sic **So**matic **An**tigen (BaSoAn) are thought to be the major components of the water-insoluble "residual" antigen (DALVADYANTS. 1997) initiating a classical T-cell-modulating state of immunity that has been protective for guinea pigs (BRUBAKER. 1972, BURROWS. 1963). BaSoAn can be obtained from *Y. pestis* and *Y. pseudotuberculosis* grown under a wide range of cultural conditions and the extraction lacks endotoxic activities (GRIBOEDOV. 1985). The B-antigen is known to be a macromolecule complex of polysaccharide, tightly bound to protein and non-toxic lipid localized on the outer coat of the virulent strains of plague pathogens. The biosynthesis of B-antigen is determined by chromosomal DNA and the optimal extraction of the antigen takes place at an incubation of temperature 27 ± 1 °C and 37± 1 °C in cultures grown on solid medium or broth commonly used in microbial laboratory. B antigen is also produced *in vivo* by *Y. pestis* (BYVALOV et al. 1997).

Subcutaneous immunized guinea pigs with B antigen were shown to be protected against similar routes of challenges with virulent *Y. pestis* in a dose of 100 $LD_{50,}$ whereby the Id_{50} was signified as 14:1.47 mg of antigen. It also initiated a classical T-cell-modulated state of the immune system (BYVALOV et al. 1997). In analogical conditions of studies, immunization of guinea pigs with 0,34 mg protected more than 80 % of the animals against aerosol infection of virulent strains with a dose ranging from 10 to 15 cfu $LD_{50.}$ In contrast to guinea pigs, B-antigen does not protect white mice (BYVALOV et al. 1997, ANISIMOV et al. 2004). Serological studies of a serum collected 21 days after immunization with F1 (Fraction-1) and B-antigen showed that there is a direct strong correlation between the level of antibody titers and survival rate of the animals, mice, guinea pig and non-human primates immunized with recombinant antigens of F1+BaSoAn were protected against challenged wild type strain of *Y. pestis.* (GRIBOEDOV. 1985).

Summing up, the native vaccines used against plague in the 20th century, killed or live attenuated and the forthcoming vaccines, based on major immunogenic antigen (F1/V) of plague microbe, known as subunit /recombinate, differences in the level of protection was observed when mice and the guinea pigs challenged by various routes. The forthcoming vaccines against plague, F1+V antigen or F1/V do protect mice but not guinea pigs and non human-primates (tab. 4) only modestly against the strains from where they were derived (TITBALL and WILLIAMSON 2001, 2004, LEARY et al. 1995). They are likely much less genetical and phenotypical diversity when compared to strains from other parts of the world, particularly FSU . Whether these vaccines protect against them is not known. Further investigation of their protection against strains isolated from various natural foci with intraspecise diversity is crucial for the qualification of any vaccine candidate against plague for global acceptance and implementation, above all in case of intentional use of the pathogen.

Although there is no comparative study between vaccines based on subunit antigens and live attenuated vaccine such as EV 76 NEIIG, it is constant that the live EV NIIEG strain provides protection for both species of experimental animals, suggesting live attenuated EV NIIEG has the advantage over the rest of the vaccine forms yet. The recombinant F1+B antigen also protect both species against bubonic plague caused by wild types and not known if it does against pneumonic plague. The recombinant antigen of F1 + BaSoAn is known only in protecting bubonic plague (tab. 4) caused by wild types (ANISIMOV et al. 2004). Further studies are essential for the forthcoming vaccine candidates to resolve the relevant characteristics that are not yet known.

Table 3. Relevant characteristics of the current and forthcoming plague vaccines in mice and guinea pigs

Protection of:												
	Mice from:				**Guinea pigs from:**				**Nonhuman primates from:**			
	w.t.		F1$^-$		w.t.		F1$^-$		w.t		F1-	
Vaccine pre	Bubonic plague	Pneumonic plague	Bubonic plague	Pneumonic plague	Bubonic plague	Pneumonic plague	Bubonic plague	Pneumonic plague	Bubonic plague	Pneumonic plague	Bubonic plague	Pneumonic plague
Live (EV)	+	+	±	?	+	+	+	?	+	+	±	±
Killed USP	+	-	-	-	+	?	?	?	+	-	-	-
F1/V or F1+V	+	+	+	+	?	?	?	?	+	+	+	+
F1+B	+	?	?	?	+	+	?	?	+	+	?	?
F1-BaSoAn	+	?	?	?	+	?	?	?	+	+	?	?
Y. pestisΔIpxM	+	?	?	?	+	?	?	?	?	?	?	?
Y. pestis yopΔH	+	+	?	?	?	?	?	?	?	?	?	?

F1$^-$: challenged with Fraction 1 negative strains, B : B. antigen, F1-BaSoAn: Fraction 1-Basic Somatic Antigens, *Y. pestisΔIpxM*- hexa-acylated lipid A mutant, *Y.pestis* yopΔH-Protein tyrosine phosphates mutant, +: Protective, ?: not known, - : not protective . Data compiled with permission from Anisimov et al. 2004, Feoderova et al. 2007, 2008, Bubeck and Dube 2007.

4.1.5 The role of major Proteins : Immunogenicity versus Virulence

The major three proteins encoded by the plasmids, *pFra, pCad, pPst,* of *Y. pestis* are well studied as single purified protein or as recombinant /subunits in order to eluicdate their role as virulence factor or protective antigens (BRUBAKER. 1991, CHERPANOV. 1991, FILIPPOVA. 1990).
By definition the ability of a biological product to stimulate an immune systems response in the body, promoting the formations of antibody is called immunogenicity, which is a desirable characteristics of an antigen for vaccine. But induction of antibody alone doesn't make an antigen to be a good vaccine candidate. It is more important to know, which antigen/ or antigens are promising, are in a position to fulfil the requirement which is necessarily needed to develop a vaccine, enable in provoking all required arms of the immune system and appropriate antibodies that must protect the vast majority of the experimental animals in the first line and finally humans from the most virulent strains of the species and have no adverse reaction, furthermore must provide universal protection against infection caused by atypical strains and retains a global acceptance (STEPANOV et al. 1997, WHO. 2004, 2007).

There are different views on the definitions of *Y. pestis* virulence determinant factors. PERRY et al. (1997) defined virulence determinant factors of *Y. pestis* as a factors that promote bacterial survival, growth, and/or transmission by directly affecting the host through adherence, thwarting host defence responses, disrupting cellular metabolism, or acquiring essential nutrients from the host. Thus, we would not classify purine biosynthetic enzymes or other metabolic enzymes as virulence determinants although they are required for virulence of *Y. pestis*, while they are common to all three human pathogen yersinae. In the contrary, BURROWS. (1957, 1960) and DOMARADISKIJ. (1998) describe that the virulence properties of *Y. pestis* as, follows: 1) the capability to form a capsule by fraction1 (F1), 2) capability of purine synthesis and, 3) formation of "pigmented" colonies on nutritional media that contain hemin.

The extreme virulence of *Y. pestis* can be attributed to its ability to efficiently invade and subvert the mammalian innate immune system resulting in an overwhelming infection. The capacity of *Y. pestis* to disarm the innate immune system is determined by numerous virulence factors encoded on its chromosome and three plasmids *pFra, pCad, pPst.* (WAKE et al.1983, BRUBAKER. 1991, CHERPANOV. 1991, FILIPPOV. 1990), which are contained by the majority of the *Y. pestis* isolates regardless of biotypes or origin has also several chromosomal associated factors that are essential to its virulence and survival in mammalian hosts and flea vectors (STRALEY and BRUBAKER 1982). Strains isolated from natural foci which lack one or two of the three plasmids are also known to be virulent by aerosols (WORSHAM and ROY 2003). The extreme virulence of *Y. pestis* to large extent, from its ability to impair innate immune system, the mechanism that enables to overwhelm the host defense, paramount among these is the plasmid, pLcrV that encodes a type III secretion system and effectors (HESSEMANN 2006, VIBOUD and BLISKA 2005). This system produces 'injectisome', that translocates *Yersina* outer proteins (Yops) to the neighbouring host cells, where

they disrupt signalling pathways, prevent cytoskeletal rearrangements, suppress cytokine, production and promote apoptosis (RUCKDESCHEL et al. 1997, HEDERSON and NATARO 2001, SMILEY. 2008, TROSKY. 2008), that indicates the system inhibits the antibacterial defense mechanism of phagocytes.

Views on virulence determinant factors of *Y. pestis* are very complex and vary. It is difficult to put a demarcation for some of the antigens of *Y. pestis* if they are very essential or not for the survival and invasions of the pathogen. One likely indicator of potential virulence for humans is an indication of high level of virulence in experimental animals. The guinea pig has been the animals of choice for virulence and immunogenicity studies in the FSU; whereas in USA or UK guinea pigs are categorized as bad model for studies of *Y. pestis* and they prefer mice as a model to study plague in general (WORSHAM. 2004). Thus different results can be achieved based on the animal species used for experimental studies and more difficult to translate results to human use. Additionally most of the studies made on the plague pathogen in the Western hemisphere are mostly based on very few strains which are very closely related with each other. Whereas, in the FSU/CIS, there are a number of strain isolates with high intraspecific diversity and equal know as virulent, but not yet known by experts outside CIS (APARIN et al. 1987, ANISIMOV. 2002).

When virulence is measured in the context of animal infection, the intensity and manifestations of pathogenicity of individual microbial strain are dependent on many conditions like animal species used for studies, immune status of the host, route of infection, infection dose, and conditions of animals care feeding (ANASIMOV. 2002, PERRY and FETHERSTON 1997).

Heterogeneity within the strains, intraspecific virulence, host specificity, biochemical and physiological traits were observed in strains isolated in FSU, Asia, and in different natural foci in Africa (MARTIN EVSKII. 1979, ANISIMOV et al. 2004). That indicates significant variability in their characteristics and virulence for mice and guinea pigs. Strain heterogeneity in *Y. pestis* can obviously have a major impact on virulence and vaccine studies. Since any studies of plague pathogenesis, focused on strains isolated in the Americas like *Y. pestis* Strain CO92, that displaying much less genetic diversity than strains found in FSU and Asia the conclusions from this studies may not be entirely applicable across the board for all strains of *Y. pestis* with high virulence for humans (ANISIMOV et al . 2004).

The FSU classification used in determining virulence differs from subspecies to subspecies. For instance *Y. pestis* subspecies *pestis* strains are lethal, whereas strains of subspecies *altaica, caucasica, hissarica, ulegica* and *talassica* as a rule exhibit dramatic reductions in virulence or even the complete absence of virulence for animals (APARIN and GOLUBINISKII. 1989, TIMOFEEVA. 1972). It is still not known which particular set of genetic and phenotypic traits are indicative for virulence in laboratory animals. At the moment, there is no one definite criterion for stating that a given strain of *Y. pestis* has high or low pathogenic potential for humans, but virulence in guinea pig and rhamnose fermentation are the two likely virulence indicators for human (ANISIMOV et al. 2004).

4.1.5.1 Iron Utilization and Pigmentation: Hms System

Y. pestis grows in fleas before being transmitted to the mammalian host, part of a cycle essential for plague's persistence. A number of proteins may contribute to the maintenance of *Y. pestis* in fleas. Among them two well-established determinant factors that play a important role for survival of the bacteria in fleas have been identified. The hemin storage (*hms*) system and the *Yersina* murine toxin (*Ymt*) (JAKSON and BURROWS 1956). The chromosomally borne *hms* locus was initially identified as being responsible for pigmentation of *Y. pesits* when cultured in vitro on agar containing hemin and Congo Red dye (PERRY. 1990, 2003). It is required for *Y. pestis* cells to colonize and block the proventriculus of the fleas. In addition in *Y. pestis*, five iron acquisition systems have been characterized (Yfe, Yfu, Ybt, Hmu and Has) and all of them are controlled by the Fur protein (THOMPSON et al. 1999, CORNIEL. 2000, GONG et al. 2001, ROSSI et al. 2001, SCUBERT. 2004). At least two (Ybt and Yfe) of them have proven to be required for the virulence of *Y. pestis* (BEARDEN et al.1998, BERDAN and PERRY 1999). One of the distinct features of *Yersinia* pathogenesis is the ability to scavenge iron from the host via siderophore called yersinabactin (Ybt). The genes of *Ybt* are chromosomally encoded and clustered on a highpathogenicity island (HPI). It was identified that eleven genes contained within an unstable 102-kb chromosomal region termed the *pgm* ("pigmentation segement"). *Pgm* is important for biosynthesis (BEARDEN and FETHERSTON 1997). It also includes the *hms* (hemin storage) locus (LILLARD et al. 1997), which confers a pigmented phenotype (greenish-brown or red) on Congo Red agar dyes plated at 26 °C but not at 37 °C. It was shown that this locus is important for transmission of *Y. pestis* by the flea vector (HINNEBUSCH et al. 1996, KUTYREV et al. 1992). Both parts are important either for virulence (Ybt) or for disease transmission *(hms locus).* Loss of virulence, linked to the loss of the capability of forming pigmented colonies on nutrient medium containing hemin, indicated a reverse and full recovery, if *a* pgm^- strain (with another virulence determinant factor) is inoculated to a mice with non-toxic doses of hemin or iron salts. But experiments in guinea pigs showed only weak effect. Replacement of iron salt with another metal was not successful. Iron did not turn a pigmented avirulent strain to virulent strain, for instance pgm^+/ $F1^+$ /VW^-, and its non-pigmented mutant pgm^- /$F1^+$ /VW^- (BURROWS and BACON 1956, 1958).

Speaking about the significance of iron ions for the virulence of plague bacilli, it is also necessary to pay attention to one circumstance, namely the capacity of all *Yersiniae* to produce new polypeptides, in an iron deficient environment. Two of them consist of high molecular weight proteins (HMWPs), synthesised *de novo*, only by highly virulent strains and are contained in the outer membrane fraction. An interesting peculiarity of HMWPs is that, all of them have a common epitops (CARNIEL et al. 1989). The essential conditions of HMWPs synthesis can be found in all organisms of humans and animals. The production of HMWPs is correlated with signs of pigment formation of plague bacilli, and deletion of HMWPs inhibits the production of macrophages to produce oxygen (O_2) radicals (DOMARADISKIJ. 1998). A chromosomal locus responsible for the pigmentation phenotype, iron-inducing proteins and iron uptake is necessary for virulence from peripheral routes of inoculation (BRUBAKER. 1991). Most of vaccine strains used as live

attenuated vaccines were pigmentation deficient (*pgm*$^{-}$), usually due to the spontaneous deletion of 102 kb chromosomal fragment encoding iron binding and transport functions. For instance, the live vaccine based on *Y. pestis* EV 76 NIIEG line, is attenuated due to the deletion of the *pgm* locus, which includes the *hms* locus responsible for the ability to store hemin and a cluster of genes needed for production of the siderophore-based yersiniabactin biosynthetic/ transport system (FETHERSON and PERRY 1994, FEDEROV et al. 2007).

Live vaccines of *pgm*$^{-}$ strains were often more immunogenic than killed vaccines in animals, but they some times cause local and systemic reactions (HALLET et al. 1973, MEYER et al. 1974). Reactogenicity varied with the host animals and the route of inoculation. For instance, such strains are virulent for mice by the intra-venous (iv) route but attenuated by peripherial routes of infection (UNE and BRUBAKER 1984), and several species of non-human primates are significantly more sensitive to *pgm*$^{-}$ *Y. pestis* than are guinea pigs (MEYER et al. 1974). Experimental studies done to examine the virulence of the *Y. pestis* C092 *pgm*$^{-}$ strain in Swiss Webster mice showed that, the strain was lethal at high s.c. dose only, with a LD_{50} of 10^{7} cfu. A dose of 10^{6} cfu (0.1 LD_{50}) of the strain given subcutaneously was generally nonlethal. The virulence of C092 *pgm*$^{-}$ strains by the aerosol route in experimental studies showed, that after exposure of 16 African green monkeys to an inhaled doses ranging from 1.1 x10^{4} to 8.1 x10^{7}cfu, eight died and eight survived, non- survivors and survivors did not differ in weight. The death was not related to the dose (WELKOS et al. 2002). Except the patho-morphological and bacteriological findings, there is no explanation to the question why half of them died while the other half survived.

4.1.5.2 Murine Toxin

The large 100 kb to 110 kbp plasmid encodes two potential virulence determinants factors: the Murine Toxin (*Ymt*) and the Fraction1 (F1) capsule antigen (CHERPANOV et al. 1991, BEN-GURION and SHAFERMANN. 1981, FILIPPOV et al. 1990, KUTYREV et al. 1986). Murine toxin was characterized as a protein fraction of the *Y. pestis* in the 1950's, and later shown to have β-adrenergic blocking ability that relates toxicity to mice and rats which is manifested by hypotension and vascular collapse when *Ymt* is released from lysing bacteria at the terminal stage of septicemic plague (BROWN and MONTE. 1977). But *Ymt* is less active in other animals such as guinea pigs, rabbits, dogs and monkeys. This suggested that *Ymt* contributes to the very low infection doses in mice, from where its name is derived (CHERPANOV et al. 2000). The pMT1-encoded murine toxin has two conserved motifs that are similar to human phosophliase D, that has shown to be located on a gene for pohospholipase D activity (RUDOLPH et al. 1999, HINNEBUSCH et al. 2000). It is localised in the cytoplasm and expressed better if *Y. pestis* is grown under room temperature. *Ymt* differs from other exotoxin, like diphteria, and anthrax toxin in: 1) there is no a latent period, 2) there is no direct relation between virulence and toxicity of the culture (DOMARADISKIJ. 1998). If not the major element, *Ymt* plays an important pathogenic role during the processes of infection of plague bacilli (EVESTIGNEEV et al. 1981). *Ymt* was shown to be required for survival of *Y. pestis* in the mid-gut of its principal vector, the rat flea *Xenopsylla cheopis*

and intra-cellular PLD activity appeared to protect *Y. pestis* from cytotoxic digestion a product of blood plasma in the flea gut (HINNEBUSH et al. 2002). When fleas were infected with *Ymt*$^{+}$ or *Ymt*$^{-}$ *Y. pestis* cells, only *Ymt*$^{+}$ cells, multiplied in the mid-gut to form cohesive aggregates and some flea was eventually blocked whereas, *Ymt*$^{-}$ *Y. pestis* cells in the flea mid-gut, did not survived (PERRY. 2003). Suggesting that *Ymt* acts from intracellular location to confer resistance to the antibacterial activity present in the flea mid-gut, which is the prerequisite for *Y. pestis* to form a blockage in flea. HINNEBUSH et al. (2002), demonstrated that *E. coli* and *Y. pseudotuberculosis* introduced into midgut of flea were rapidly eliminated or reduced in number. However, when either of these two species was transformed with recombinant *Ymt* gene, survival capacity in fleas dramatically increased. Therefore, Ymt is essential for the maintenance and survival and multiplication of *Y. pestis* in flea.

Recent observation by VASILEVA et al. (2005) indicate that Mouse toxin (MT) was found to have a pronounced apoptogenic action with respect to the phagocytic cells of mice, but not guinea pigs. Macrophages were affected by this action stronger than neutrophils, and in both cases this effect was dose dependent. As the dose of MT decreased to 0.01 μg/ ml, the proportion of cells dying as the result of apoptosis increased, the necrotic type of damage was almost absent. On the contrary, as MT concentration rose to 1.0 microg/ ml and over, the proportion of phagocytes dying due to necrosis increased with a decrease in the number of cells in which the process of apoptosis started. The results of the study are indicative of the fact that the mechanisms programming the death of cells under the action of MT on macrophages and neutrophils took part in the process, which, in its turn, determined their role in the pathogenesis of plague.
To determine the protective efficacy of *Ymt* as it was included in the production of chemical vaccine, a 100 to 500 μg purified Ymt *per se* with incomplete Freund's adjuvant or aluminium hydroxide did not protect against sc inoculation guinea pigs and mice against LD_{100}. There were no sign of development of antibacterial immunity (EVESTIGNEEV et al. 1981). These factors make *Ymt* a weak candidate to be used as a recombinant antigen or chemical vaccine production against plague.

4.1.5.3 Fraction I

BAKER et al. purified the specific capsular like antigen of *Y. pestis,* Fraction 1 (F1), in 1952. It is the major component of the surface of the plague bacilli that is expressed at 37°C on nutrient media. However, not at a temperature of 26 °C-28 °C (optimal growth temperature of *Y. pestis*) where, the F1 is not expressed or weakly expressed (FEODOROVA and DEVADARIANI 2001).

The genetical control of F1 synthesis is encoded by a unique 100 -110 kbp large plasmid called (pFra). Its molecular weight ranges from 60 to 65 mDa. F1 forms a large gel-like capsule containing multimeric aggregates (BENNETT and TONABENE 1974). Production of the 15,5-kDa F1 subunit is encoded by the *caf1*, *caf1M* gene (GALYOV et al. 1990, 1991) and the *calfA* (KARLYSHEV et al. 1992) genes that encode, respectively, a chaperone which allows export of the F1 antigen subunit to the surface and a anchors protein which the F1 antigen into the outer membrane. The essential

carbohydrate component of mature FI antigen was suggested to be encoded by chromosomal genes, which were not specific for *Y. pestis,* but were found also in all *Yersinia* spp. and some other bacteria (FEODOROVA and DEVADARIANI 2001).

Experimental investigation of pFra mutant strains of *Y. pestis* from the Year 1991-1996 found, that *fra*$^{-}$ (mutants) which were unable to synthesise the chaperone *caf1M* were capable of forming a capsule, which was different from the wildtype and had different virulence, level of protection, biochemical characteristics like hydrophobicity and isoeletrical potential (IS). *Caf1M*$^{-}$ mutants of virulent strains of *Y. pestis* were able to cause an infection that was similar to the "classical" plague infection clinically and histopathologicaly. But it was unable to protect, against homologous strains (ANISIMOV et al. 1997).

Y. pestis M493 isolated from a natural focus and stored as "Fra±" (strains which were observed in producing F1 only in destructed cells but not in nutrient medium) for several years in the museum of „Microbe" showed atypical capsule formation, i.e it was unable to form a capsule (PROZENKO et al. 1983, BAKANURSKAJA and NEKRASIVA 1997). This indicates the possibility of essential structural changes in the area of the *caf*-gene. Analysis of the clon of lyophylized and culture grown on hottinger agar showed in 10-30 % of cases lost pFra and in 8-25 % of the cases the clon increased molecular mass of pFra in comparison to "wild "type strains (SHEREMET et al. 1987, BAKANURSKAJA. 1997, ANISIMOV. 1999). Serial passages of heterogen culture through organs of white mice resulted in deletion of atypical clon population from the plague microbe. Atypical strains were characterised, strains with identical morphological and biochemical properties to the "classical" capsule forming strains but differ in immunological tests. In experimental model of white mice (Balb/C) infection with atypical strains showed resistance against immunization with live attenuated EV vaccine NIIEG line, suggests that pFra mutation does not directly result in avirulence (ANISIMOV and MARKOV 1997). This is an indication for mutation that took place through long period of storage, or for new essential antigenic variation. The F1 subunit is thought to be assembled on the surface of the bacterium to form the capsule, where its production is temperature regulated by the product of the *caf1R* gene (KARLYSHEV et al. 1992). A plasmid with molecular weight of 13 mDa, which is found uniquely in the strains of Yerka might take part of F1 synthesis (TSUKANO et al. 1986). In addition, four more extra plasmids were expressed by this specific strain (TSUKANO. 1989, ZHAO et al. 1990) this indicates that F1 synthesis is controlled by chromosome and it is responsible for protection. The F1 gene was found to code for 17,5-kDa polypeptide carrying a putative secretion signal (GALYOV et al. 1990). It is considered to be an important but not essential virulence factor unique to *Y. pestis* (DAVIS et al. 1996, WELKOS et al. 1995). Deletion of the F1 gene does not abolish virulence but leads to delay in onset of the disease in animal model. Oral infection of 98 outbred mice with virulent *Y. pestis* strain 358 (pFra^{-}) showed reduced virulence to 100 folds as in compared to its isogenic variant that harbors all three types of plasmids known in plague microbe (KOKYSHKIN. 1997). It is even possible to propose, that the product of the virulence determinate factor of plasmid pFra is necessary to realize the pathogenic process of plague infection caused via alimentary tract. F1 appears to have a role in blocking uptake by macrophages (ROSQUVIST and FORSBERG 2002), it also prevents the association with

phagocytes preventing uptake by interfering at the level of receptor presumably adhesin-receptor interactions in the phagocytosis. Thus, F1 antigen inhibits phagocytosis (DU et al. 2002).
At the moment any discussion of plague vaccines would be in complete with out consideration of Fraction 1 (F1). The specific capsular antigen of *Y. pestis* is highly immunogenic in both, animals and humans, and is widely used in serological tests for detection of specific antibody to *Y. pestis.* F1 is the principal immunogen in killed or live attenuated vaccines which were widely used in different epidemic regions of the world, which showed efficient protection against bubonic plague. However, it is less active against the pneumonic form (BARTELLONI et al. 1973). Although some adverse clinical reactions were observed when F1 is used as a vaccine alone, it is one of the best antigen candidates in order to develop vaccines against the plague microbe in any possible form of construction.

4.1.5.4 V-Antigen

The **L**ow-**c**alcium **r**esponse **V**irulon or **S**timulon (termed pLcrV, pCad, pVW or pYV) is a 68 to 75 kb plasmid that encodes a major groups of defined virulence proteins. V-antigen of *Y. pestis* encodes the leading virulence factors , the type III secretion system (T3SS) complex (CORNELIS et al. 1998, PETTERSON et al. 1999, FIELDS et al. 1999) and effectors known as Yops Yersina outer proteins (PERRY and FETHERSTON 1997, GHOSH. 2004). Host-pathogen interaction result in expression changes within both the host and the pathogen. Although pathogenic Yersina species are predominately an extracellualr, they can translocate heterologus antigens from extracellulaur location to the cytosol of the APC with their type three secretion system (T3SS) which are critical for virulence to evade the host. To overcome host defense mechanisms, yersiniae translocates a set of Yops into immune cells to inhibit the innate and the adaptive immune system. At list four Yops (YopH, YopE, YopT and YopO/YpkA) are involved in inhibiting phagocytosis of yersiniae by disrupting the cytoskeleton of PMNs and macrophages (ROSQVIST et al. 1990, 1991, SCHESSER et al. 1998, BLISK. 2004, ZHOU. 2006).

A comparative analysis of the structure of the pYV from 14 strains of *Y. pestis* and *Y. pseudotuberculosis* showed that they possess identical *BamHI*- (B) and *HindIII*-sites (H) in the LCR-region, which proofed that they are highly conserved and homologous in two other pathogenic species, *Y. psedotuberculosis* and *Y. enrtercolitica* as well (BOBROV and FILIPPOV 1997, BÖLIN et al. 1982). The plasmid of *Y. pseudotuberculosis* (pYV) differs from that of *Y. pestis* in that, it lacks one or two B-sites in the YopkA/YpO gene region, and an integrative mobile elements, *IS100* which is found only in pCad of *Y. pestis* and *IS285* which is found in *pcad* of *Y. pseudotuberculosis,* predominantly (BOBROV and FILIPPOV 1997). These might be the product of mutations in the process of evolution and a reflection of phylogenetical relation of the plasmids. Maximum expression of the V-antigen and YOPs occurs in vitro at 37 °C in the absence of calcium or low-calcium-media (STRALEY et al. 1993) or after an intimate contact of the bacteria with the plasma membrane of eucaryotic target cells (ROSQVIST et al. 1991, SORY et al. 1995).

Antibodies rose against purified preparations of V-antigen provided protection against plague in mice and polyclonal antibodies rose against highly purified LcrV as well as monoclonal antibody that recognize the central domain of LcrV conferred passive protection against experimental infections with *Y. pestis* and *Y. pseudotuberculosis* (LAWTON et al. 1963, UNE and BRUBAKER 1984, MOTIN et al. 1994, HILL et al. 1997). It has been established that active immunization using LcrV as an antigen can provide high level of protection against up to 10^7 cfu lethal doses in experimental bubonic and pneumonic plague (LEARY et al. 1995). Similar results were observed during studies done on immune responses of mice survived from pneumonic plague. Over 90 % of mice immunized with V antigen showed an immuno-globulin (IgG) titers with an average of 1:9,000 measured by ELISA and immunoblotting methods (ANDREWS et al. 1999). Recombinant V (rV) antigen vaccine adsorbed in to aluminium hydroxide protected mice against 7.4 x 10^5 LD_{50} $F1^+$ as well as $F1^-$ strains of *Y. pestis* (ANDERSON et al. 1996).

Comparative sequencing of the V antigen gene from different geographical origins showed that the very majority of the strains posses' identical nucleotide sequences. The lack of *lcrV* differences among these strains suggested that little evolutionary distance separates them from a common ancestor, with only few exceptions such as the Angola strain (PRICE et al. 1989). This fact is of major concern in considering rV-antigen as candidate for vaccine. If that is also the case in strains isolated from natural foci in FSU, Mongolia and China is not yet known. There are strains isolated in FSU, with different sizes of *pCad*, strains with additional cryptical plasmids isolated in different regions of the world.

Previous studies by SATO et al. (1991) and LAWTON. (1963) demonstrated a clear role of antibodies to the V antigen in passive protection, though the mechanism by which the V antibody prevents fatal disease is not yet clearly explained. A good IgG antibody response to the rV antigen developed as early as 13 days post immunization and its titers continued to rise up to the time of challenge was observed on day 58 post immunization. This can be the bases of its efficacy. The predominant IgG subtype was shown to be IgG1, followed by IgG2a and IgG2b when aluminium hydroxide is used as adjuvant (GEORGE et al. 1996). The ability of rV to prevent death and the establishment of a chronic infection in the majority of the surviving mice challenged subcutaneous or by aerosol indicates the value of this immunogen as a part of any subunit vaccine against plague. However, it has also been proposed that LcrV acts by suppressing the inflammatory responses early during infection, suppress the innate immune response, alters cytokine profiles during *Yersinia* infection, which may contribute to immune system subversion (BRUBAKER. 1991, NAKAJIMA et al. 1993, 1995, NEDIALKOV, et al .1997). This is supported by experiments in which the injection of LcrV in mice suppressed the expression of TNF-α and IFN-γ and promoted the survival of LcrV-negative *Y. pestis* (NAKAJIMA et al. 1995). LcrV inhibits neutrophil chemotaxis *in vitro* as well as *in vivo* (WELKOS et al. 1998). In addition to its effect on host V protein is involved in the regulation of the low calcium response of *Y. pestis* (BERGMAN et al. 1991, PRICE et al. 1989, 1991, SKRZPEK and STRALEY. 1995). This limitation could damper the ability of a vaccine using a combination of recombinant LcrV and F1 subunit (WILLIAMSON et al. 2001) separate or as a fusion protein (HEATH. 1998). In addition to that, it is also not known, how the recombinant

vaccine formulation responds to strains with various plasmid contents and strains isolated from natural foci, which lack the LcrVantigen (DMITRYUK et al. 1997). Nevertheless, experiments performed with mice illustrated the efficacy of the V antigen as a vaccine against lethal subcutaneous and aerosol infections with $F1^+$ and $F1^-$ *Y. pestis* strains (ANDERSON et al. 1996, HEATH et al. 1997, LEARY et al. 1995, MOTIN et al. 1996, WILLIAMSON et al. 1995). Althugh, the V antigen of the *Y. pestis* has been described to the potent suppressor of TNF production, the pro-inflammatory cytokine which is a mediator of inflammation and plays a major role in host responses to microbial infections (VASSALLI. 1992). TNF is predominantly produced by activated macrophages, although other cell types, e. g. T cells, NK cells and the mast cells have been recognized as sources for TNF. Locally, TNF acts as a stimulator for the expressions of cytokines including IL-1 and IL-6, and chemokines such as IL-8 and monocyte chemotactic protein-1MCP-1 (LI, 1993, SCHULTE et al. 1996). TNF also induces the expression of adhesion molecules E-selectin, vascular cell adhesion molecule-1 and stimulates macrophages to produce large quantities of nitric oxide, which is thought to contribute to killing of bacteria (EVANS. 1995). Comparatively, little is know about countermeasures of the TNF responses.

The V antigen is also characterized as suppressor of TNF and IF-γ levels in sera and spleens of mice infected with virulent *Y. pestis* when compared to control mice (ANNET et al. 1999). That indicatres that V-antigen is an interesting protein, because once it is not only required for the translocation process, but it has also been detected in the cytoplasm of the host cells and the extracellular milieu of the infected cell (BLISKA. 2004). It is also known as one of the major immungenic component of *Y. pestis*, a candidate for new vaccine development and on the other side it is also proofed as immune suppressive protein, it counteracts cytokine production that might cause toxicity after immunization. Currently attempts to neutralise the suppresive effect of V- antigen are under studies. However V antigen is the next strong candidate protein in development of plague vaccine to F1 in any form of vaccine design.

4.1.5.5 Pesticin

Pesticin was first isolated from plague bacilli by BEN-GURION and HERTMAN in 1958. Three years later, BRUBAKER and SURGALLA reported that the plague microbe possesses additional pesticin, and then they named it P1 and P2, respectively. The majority of plague microbes posses P1, but not *Y. pseudotuberculosis*. P2 is characteristically produced by both, *Y. pestis* and *Y. pseudotuberculosis*. The principal difference between them is, that the production of P2 is coded on the chromosome, whereas a gene of p1 "*pst*" is localized on the plasmid P (*pst*), it is also known as (pPCP1) with molecular weight of 9.5 kb, the smallest plasmid harbored by *Y. pestis* (DOMARADISKIJ. 1998).

Pesticin (P1) of *Y. pestis* is determined by the pesticin activity gene (*pst*), [40 kDa] and the immunity gene (*pim*) [16 kDa] found in periplasma (PILSL et al. 1996). The genes are tandemly arranged on small plasmids and transcribed in opposite orientation, which is typical for pore-forming colicins, as opposed to the colicins with nuclease activity (BRAUN et al. 1994, JAMES. 1996). Both pesticins neutralize antiplague serum and virulent strains of *Y. pseudotuberculosis*. Pesticin was considered as a *N*-acetyl-glycosaminidase previously, and apparently kills by degrading cell walls leading to osmotic instability and lysis (FERBER and BRUBAKER 1979). VOLLMER et al. (1997) indicated that pesticin is a muramidase and not a N-acetyl-glucosaminidase, that converts cells into stable spheroplasts by slowly degrading murein. Pesticin 1 was reported to be essential for the virulence of plague bacilli previously, as variants of *Y. pestis* lacking pesticin *(pst-)* were avirulent in comparison to strains that possess pesticin 1 in white mice and guinea pigs challenged subcutaneously. But intraperitoneal challenge reduced its virulence to 4-5 folds, and both $pst1^-$ and $pst1^+$ strains were similar in intravenous inoculation. Further investigation of the mutants showed that they lack coagulase and fibrinolytic activities (BRUBAKER et al. 1965). In contrast, GREBTSOV et al. (1991) reported that pesticin do not play an essential role in expressing virulence and immunogenicity, but it is possible, that a vaccine strain of *Y. pestis* circulating in the organism can induce antibodies and can contribute to protection of the animals from infection. Similar investigation also showed that all mutants of plague microbes, that lost the capability for P1 production, were avirulent, although some of avirulent strains produce P1 (DOMARADISKIJ. 1998).

Guinea pigs infected with *Y. pestis* strain 358 that harbor $pPst^+$ and its mutant $(pPst^-)$ do not lead to an increase in LD_{50} with either in s.c. or respiratory challenge (SAMOILOVA et al. 1996). Another similar investigation showed that, six white mice and six guinea pigs challenged s.c. with the doses ranging from 10 to 10^4 cfu/per animal with *Y. pestis* 231 strain that harbor pesticin (pst^+) and its mutant (pst^-) did not show significant difference in both groups of animals, indicating that pesticin alone is not essential as factor of virulence in plague microbe. Similar studies on its immunogenicity showed no signs of protection (GREBTSOV. 1991 Some minor differences were observed in KIM and CO92 and some other strains. Although it was observed that *Y. pestis* strains which lost their capacity in producing *P1* or the "switching-off" of *pst* gene had decreased the level of virulence i.e. increased the mean survival rates of the animals to two days longer than the wild

strain, significant level of protection was not observed when white mice and guinea pigs were s.c. inoculated. Some mutant (*pst*-) strains isolated from natural foci have been reported to be virulent (APARIN et al. 1987)

Even though pesticin antigen alone is thought to be a weak immunogen, In Russian standard criterieum, like the live EV 76 NEIIG, the paternal vaccine strain, the forthcoming new vaccine strains candidates for the development of improved live attenuated vaccine must harbor pesticin (ANISIMOV et al. 2002). The reason might be, that pesticin may play some roles in concert with other immunogenic proteins like F1 and V-antigen in processes of inducing humoral or cellular immnuesystems that needs futher studies.

4.1.5.6 pH 6 Antigen

The pH 6 antigen (PsaA) is the only known putative adhesion antigen expressed on the surface of *Y. pestis,* when the bacterium is grown between pH 5 and pH 6.7 and between 35 °C and 41 °C encoded by the chromosomal *psaA*, while cells grown outside this parameter did not (PRICE et al.1995). The *psaEFABC* operon encodes a chaperone/usher pathway involved in the secretion and assembly of pH6 antigen as polymers on the surface of *Y. pestis* macrophages. These conditions are encountered in the phagolysosome and synthesised at this site in macrophages, which might facilitate the colonization of other cell types and contribute to the delivery of Yops into these cells by promoting bacterium-host cell contact (LINDLER et al. 1993, ZAVYALOV et al. 1996). The pH 6 antigen was first identified as a virulent determinant factor when the derivatives of *Y. pestis* lacking the pH 6 antigen showed reduced virulence in mice (BEN-EFRAIM et al. 1961). Mutation of the *psa* locus results in a 200-fold increase in the LD_{50} of the mutant bacteria compared with the wild-type parent strain, when mice were challenged intravenous route of infection (LINDNER et al. 1990). pH 6 antigen binds cerberosides containing ß1-linked galactosyl residues in glycosphingolipids of the host and are likely to be found on range of host cell types recognized by pH6 antigen. Thus a role of the pH6 antigen might be to maximize the colonization of the host cells (PAYNE. 1998). However, HUANG et al. (2004) demonstrates that pH6 antigen did not enhance adhesion of the bcateria to mouse macrophages but rather functioned as antiphagocytic factor independent of Yops and F1 capsule. Therefore, pH6 antigen is unlikely to be adhesin that was previously thought to be responsible for the pathogen's colonization of of host cells or the delivery of Yops to the host cells.

The pH 6 antigen is induced inside macrophages under acidic environment a homopolymeric fibrillar structures on the bacterial surface (LINDLER et al. 1993). Production of the antiphagocytic of *Y. pestis* cells is reduced or not expressed under this condition, where the expressing of the pH 6 (psaA) antigen is optimal (LINDNER et al. 1990). Suggesting that pH6 antigen might be the first antiphagocytic factor produced by the organisim after release from infected macrophages which is specific to pH6 antigen expressing organism, different from that of Yops (MAKOVEICHUK et al. 2003). It was also found that pH6 aggultinate erythrocytes from a range of species and the pH 6 antigen has been reported to bind to several human immunoglobulin G subclasses by acting as a

bacterial Fc receptor (ZAVYALOV et al. 1996). Intradermal injection of pH6 in guinea pigs isolated in immunosorption method induces inflammatory reaction and possesses hemagglutnating and cytotoxic activities in alveolar macrophages (STEPANSHINA et al. 1993).

Immunological responses of 12 mice after aerosol challenge with approximately 100 LD_{50} of *Y. pestis* CO92 and subsequently treated with antibiotics starting 24 or 42 hrs post challenge showed no reaction. Thirty days post-challenge collected sera from survivors analysed for IgG by ELISA and immunoblotting methods specified that only one (1/12) animal responded to pH 6 antigen (BENNER et al. 1999) that indicates pH6 is a weak immunogen inductor to be considered as a candidate for subunit or recombinant vaccine production against *Y. pestis* alone.

4.2 Challenges of Vaccine Development against Glanders and Melioidosis

In the last century the world had witnessed two world wars, where one of the oldest know pathogen that causes glanders, which is now known as *B. mallei* was used as biological weapon (GEISSER,1998). As a consequence, a number of states further intensified the research activities in quantities of microbes considered most suitable for weaponization during cold war era (BONDI and GOLDBERG 2008). The very majority of research activities of these pathogens were done with high security, secrecy in military centers and moreover these pathogens were genetically manipulated to enhance the resistance against any forms of antibiotic therapy or vaccines in some states (ALIBEK. 1999).

B . mallei is an obligate parasite that typically infects solipeds and only occasionally infects humans, whereas *B. pseudomallei* is a widely distributed environmental saprophyte that cause a severe disease in human and animals in endemic regions (DANCE. 1991, WHITE. 2003). Because of high mortality rate in humans and the small number of organisms required to establish infection, if inhaled and furthermore, high MDR to most fortline antibiotics, relapses after complete therapy and intrinsic resistance *(B. pseudomallei*) in the environment, and lack of vaccine, they have been classified as category B potential candidates of biological warfare or bioterrorism agents (CDC, 2000, KONDRIK et al. 2003), that enforces the development of effective prophylactic vaccine, to be the most realizable approach to the solution. Although infection with *B. mallei* and antibiotic therapy was raerly known, a single reported case know in human glanders, was laboratory related infection of a researcher n USA that showed the difficulty of the course of different antibiotics and its relapses (SIRINIVASAN. et al. 2001) indicating that, *B. mallei* might have similar characteristics of resistance to antibiotic therapy as its closely related MDR *B. pseudomallei* (BATANOV. 1997).

The recently complete sequenced genomes of *B.mallei* strain ATCC 23344(NIERMAN et al. 2004) and *B. pseudomallei* K96243, a strain isolated in 1996 from a 34-year–old female diabetic patient in Khon Kaen hospital in Thailand (HOLDEN et al.2004) promoted research activities in determining virulence factors, pathogenesis, relationships and intraspecific diversity with in the strains and species. *B. mallei* is a clonally related organism to *B. pseudomallei,* as the genomes were compared, *B. mallei* is smaller with 5.8 Mb than *B. pseudomallei* with 7.5 Mb or *B. thailandensis* (environmental soil inhabitants) with 6.7Mb. Comparing the genome with one another, the authors found that 627 genes in chromosome 1 and 819 genes in chromosome 2, as one, 1446 genes of *B. pseudomallei* are either not present or variant in *B. mallei.* Whereas common genes to both species were highly homologous and organized similarly along the genome. Indicating the possibility of developing a single vaccine against both pathogens, most predictable an efficient melioidosis vaccine may provide protection against Glanders. Vaccine development will also profit from the current molecular tools, where by immunogenic substance can be screened and rationally designed and further studied in animal models.

Experimental Studies of vaccine development against glanders and melioidoise diseases includes, Syrian golden hamster, BALB/c mouse, C57B6/ mouse, CBA mouse, Guinea pigs, White rats, and monkeys, which are differently susceptible to the disease (KALACHEV et al. 1997, MANEZENYUK et al. 1999, ILYUKHIN. 1999, PIVEN. 2001). Syrian golden hamster are the most common animal model used for studies, but they are extremely sensitive to virulent strains of *B.mallei* and *B.pseudomallei, (*LD_{50} of 1-10 microorganisims when challenged s. c., and intraperitoneally with *B. mallei* C-5 and *B. pseudomallei* C-141), even a single cell can cause the disease which is characterized by acute septicemia just within an hour after infection. Guinea pigs, CBA mouse and Monkeys were found to mimic a chronic infection (MANEZENYUK et al. 1999).

Experimental studies of BALB/c mice mimicking for acute Burkholderia infection made, showed acute clinical symptoms and succumb in only three days, while the C57B/6 mice showed heterogenic chronic symptoms with the same infection dose and can survive for two to six weeks post infection was reported, indicating CS7B/a shows a moderate resistance in comparison to BALB /C Mouse model (BRETT and WOODS 2000). It was reported that BALB/C mice are susceptible by a factor of 10 to 100-folds when compared to C57B/6 mice independent from the portal entry of infection (TAN et al. 2008) Similar clinical manifestations are also known in human infected especially with melioidosis. The question is not yet elucidated why some of the infection are acute where as others are sub acute or chronic and relapses were observed after 26 years of period in human. It seems, that next to the route of infection, the status of the immune system of the host must have some role to play as in the case of various mouse models.

Although basic research activities on these pathogens have made progresses in the last decade, study of the immune responses against pathogenic *Burkholderia* species are complicated by the lack of animal models which survives long enough for the immune system to clear infection. The genomic plasticity of both pathogens, even within the type strain and the capability of residing in eukaryotic cells moreover cause difficulties in vaccine research against both pathogens (HOLDEN et al. 2004,NIERMAN et al. 2004;PRUKSACHARTVUTHI et al.1990). However various forms of approaches were made in attempt to develop vaccines, that includes killed whole cell vaccines, live attenuated vaccines, heterologous vaccines, acellular vaccines, subunit /recombinant DNA vaccines in various species of animal models (DANNENBERG and SCOTT 1958, LEVINE and MAURER 1958, VASILEIV et al. 1993, MANEZENYUK et al. 1999, ILYUKHIN et al. 2002,AMEMIYA et al 2002, WHITLOCK et al. 2008).

4.2.1 Killed Whole Cell Vaccines

The New York City board of health conducted an experimental immunization with a suspension of dried glanders bacilli, which was prepared in their laboratory in guinea pigs and horses in 1913 (MOHLER and EICHHORN 1914). Guinea pigs received three weekly injection with the suspension containing 2mg/ml, subcutaneuosly. However none were resistance to the challenge. In the experiment conducted on 17 glanders-free, most of them aged, but in fair conditions, three or four injections with a dose escalating from 1ml to 12 ml were used. One week after the last vaccination, two horse were infected with *B. mallei* via nasal mucous membranes. Both contracted glanders and one died of acute form of the disease 21 after the infection. The rest of the animals were exposed to natural forms of infections, and four of the animals developed the diseases within 8 months. Nine out of 13 horse contracted glander diseases naturally. Thus the Authors concluded that control and eradication of glanders was dependent on elimination of infected horses and prevention of infected horses from entering stables that were free from diseases. Although these initial study and proposal of the Author is almost a century old, currently similar measures are used in areas where glanders disease outbreak is known. For instance, in spring 2007 outbreak of glanders in Russian auotomous Chita region remind us that 100 horses which were tested positive to glanders disease and all horses in-contact with infected animals were been killed, so far there is no vaccine.

AMEMIYA et al. (2002) evaluated three nonviable cell preparations of *B. mallei* plus Alhydrogel in a, a heat-killed, an irradiation-inactivated and an irradiation-activated capsule mutant strain preparations BALB/c murine model of glanders. None of them provided protection against lethal challenge. It was reported that all three preparations yield similar immune responses when spelonocyte proliferation and sera immuno-globulin were analyzed and expressed various cytokines like IL-2, IFNγ, and small amounts of IL-4 and IL-5, and more IL-10. The author found higher level of IgG1 than Ig G2 subclasses production and, a mixed T-helper-cell like responses, demonstrated by Th1- and Th2-like cytokine and a Th2-like subclass Ig responses. Partial protection was observed when BALB/C mice were immunized s.c., with a mixture of nonviable *B. mallei* cell with IL-12 against i.p. challenged doses of (143 LD_{50}s) as independently. It also showed that the vaccine enhanced predominantly IgG2a isotype level and significantly increased the proliferation activities and IFNγ production by splenocytes. It was also reported that the spleen of vaccinated survivors were generally enlarged and heavily infected with *B . mallei* (AMEMIYA et al. 2006).

Attempts of passively transferred immunity with immune serum were unsuccessful (KOVALEV. 1971). Passive transfer of monocolonal antibodies specific for *B. mallei* have also demonstrated early protection of mice infected with lethal aerosol challenged *B. mallei* (TREVINO et al. 2006). To determine the mechanisms involved in the development of protective immunity in a murine model of melioidosis BARENS and KEETHESAN (2007) reported that, C57BL/6 and BALB/c mice intravenous infection with *B. pseudomallei*, demonstrated delayed-type hypersensitivity (DTH) responses and the generation of *B. pseudomallei*-specific lymphocytes towards *B. pseudomallei*

antigens. No protection was observed when mice were subsequently challenged with lethal doses of highly virulent strain of *B. pseudomallei*. Subcutaneous immunization of BALB/c mice with live *B. pseudomallei* vaccine demonstrated significant protection compared with exposure to heat-killed *B. pseudomallei* culture filtrate. cell culture. In contrast SARKAR-TYSO (2009) recently reported that heat-inactivated *Burkholderia thailandensis*, *B. mallei* or *B. pseudomallei* cells as vaccines against murine melioidosis and glanders provided efficient protection against intraperitonial as in aerosol challenged with homologous strains and some cross protection were also observed when the mice were challeged with hetrologous strains. The Author explains the protection of heat killed vaccine in this study, might related to the much higher number of killed bacteria cells (10^8 cfu) used and differences in routes of administration.

4.2.2 Live Attenuated Vaccines

The resistance of mice and hamsters vaccinated with live avirulent strains of *B. pseudomallei* against aersol–initiated melioidosis with virulent *B. pseudomallei* strain 103–67 in comparison to non immunized mice were reported by DANNENBERG and SCOTT (1958, 1960), however the vaccine did not prevent the progress of the disease. Similar results were also reported by LEVINE and MAURER (1958).

ILYUKHIN et al. (1999) investigated the efficiency of selected attenuated auxotrophic mutants of *B. pseudomallei*, purine dependent (*Pur*-) strains of *B. pseudomallei* (C141-206, 60806-10, 57576-25, 60806-90) (Museum of Live culture Collection of Volgograd research Institute for plague control, Russia) (MERINOVA. 1997) and thermo-sensible (T^s) strains of *B. pseudomallei* (57576-6, 57576-7, 57576-9) vaccines in four species of animal models that includes Syrian golden hamsters, White rats, Guinea pigs and BALB/mice. Immunized animals showed significant resistance against s.c. challenged doses 1DLM (100 LD_{50} for Golden hamsters and 1000 LD_{50} for the rest of experimental animals) of highly virulent *B. pseudomallei* strains. The author found that, the best protective was obtained by the vaccine based on *B. pseudomallei Pur* - mutants (C141-206, 60806-90) and thermo-sensible (T^s) strain 57576-9 and the survival rates of immunized animals in average increased from 4 to 7 days in comparison to the control groups (tab. 4). But s.c. immunization of eight guinea pigs with *B. pseudomallei* strains C141-206, 60806-90 and T^s 57576-9 did not protect any of the animals from aerosol challenges, all animals died after aerosol infection of 1DCL with virulent *B. pseudomallei* 57576 strain. That in fact indicates the level of protection of the vaccines depends on the the route of infection induced.

Table 4. Survival of animals immunized with live attenuated vaccines of *Pur-, T^s* strains of *B. pseudomallei* against subcutaneous infection induced melioidosis

Immunization	Infection	Animals	Mtd	Survivors /total	% protection
B. p. C141-206	B.p. C141	Golden hamster	2	4/16	25
B. p. C141-206	B.p. C141	BALB/ mice	14	10/14	71
B.p. C141-206	B.p. C141	Guinea pigs	7	3/10	30
B.p. C141-206	B.p. C100	White rat	4	4/9	33
B.p. C141-206	B.p. C141	White rats	17	4/9	33
B.p. 60806-10	B.p. C41	BALB/mice	5	3/10	30
B.p. 60806-90	B.p. C141	BALB/ mice	3	6/12	50
B.p. 57576-25	B.p. C141	BALB/ mice	7	2/12	16
B.p. 57576-9	Bp. C141	Guinea pigs	2	9/14	65
B.p. 57576-9	B.p. C141	Hamster	1	1/9	11

Notes**.** Immunization B.p- *Burkholderia Pseudodomalle*(Collection of the Museum of Live Culture of Volgograd, Russia), MTD-Mean date to death in days. Table compiled from Ilyukhin et al. 1999.

Mice immunized with live attenuated *B. pseudomallei* purine auxotroph strain were also reported to be protective against the challenged wild-type of *B. pseudomalli* strain. But in light of the considerable antigenic variation present within *B. pesudomallei* strains (ATKINS et al. 2002).

ULRICH et al. (2005) studied the ability of aerogenically vaccination with two live attenuated *B. mallei* strains, (a capsule mutant and a branched-chain amino acid auxotroph) to protect against high doses of (300 LD_{50}) aerosol-initiated glanders in mice. Analysis of serum samples obtained from mice immunized with capsule mutant strain developed a Th2-like Ig subclass antibody response. It was found that the immune response did not provide protection, as no mice challenged with aerosolized *B. mallei* survived 5 days after infection survived. On the other hand mice immunized with auxotrophic mutant elicited a Th1-like Ig subclass antibody response that correlates with 50 % protection against a lower dose (5, $0x10^3$: 5 times LD_{50}) aerosolized challenges. It was also shown that serum IgG and IgM titers of mice were 20 and 60 fold higher than that of naive controls. However 10^5 cfu bacteria/ gm from organ homogenates i.e. spleen, lung and liver were counted.

Previous studies showed that the immunological response that protects from pathogenic Burkholderia species were determined by the T- immune system (JONES. 1980). Further, studies in a murine model of melioidosis by KETHEESAN et al. (2001), also showed that the development of a T cell response in C57BL/6 mice infected with *B. pseudomallei.* In addition, *in vitro* a high proliferation response of purified splenic T cells from infected mice was observed in the presence of *B. pseudomallei* lysates. Similar studies carried out on 12 patients recovering from melioidosis

demonstrated the development of specific T cells responses and the involvement of both, CD4+ and CD8+ cells, in response to *B. pseudomallei* antigens. However, recent studies showed that both humoral and cell-mediated adaptive immune responses are required for whole protection against *B. pseudomallei* and *B. mallei* challenge and for bacterial clearance post-infection (HEALEY et al. 2005). That indicates a vaccine which is protective against both pathogens must provoke both arms of the immune systems. For facultative intracellular organisms like *Burkholderia,* a live attenuated vaccine may be the best strategy of choice to induce both, cellular and humoral immune responses; they mimic natural infections and generate systemic and mucosal immune responses. They can often induce the production of cytokines that recruit the elements of the immune system that ordinarily do not responds to subunit and inactivated or killed vaccines (ULETT et al. 2000, KEETHESAN et al. 2002).

Immunization based on avirulent strains of *B. thailandensis* (10^8Cfu) demonstrated more than 50 % of protection in guinea pigs challenged s.c. with 200 LD_{50} of *B. pseudomallei* 100 virulent strain investigated for 60 days, where as all control groups died with in 2-3 weeks. Guinea pigs immunization based on *B. cepacia* strains did not show significant protection (ILYUKHIN et al. 2002). That suggests vaccine based on *B. thailandensis* might be considered as an alternative prototype candidate. *B. thailandensis* is avirulent and is closely related to both pathogenic *Burkholderia* species, thus it is possible to say vaccine based on *B. thailandensis* (tab. 5) will be one of the prototype of vaccine candidate which needs further investigations for human use.

Table 5. Protective efficacy of vaccine based on *B. thailandensis* against Melioidosis in guinea pigs

Strains		**№ animals immunized**	**% protection**	**MTD (days)**
Immunization	**Infection with (cfu)**			
B. thailandensis 251	B.p.100 (2x 10^3)	7	57	22
B. thailandensis 265	B.p.100 (2x 10^3)	11	64	26
B.cepacia 25416	B.p. 100 (5x10^3)	12	17	24
Control	B.p.100 (2x 10^3)	5	0	16

Notes. B.p - *B. pseudomallei*, cfu- colony forming units, MTD- Mean time to death in days, compiled from ILYUKHIN et al. 2002).

4.2.3 Heterologous Vaccines

For instance immunization with Live Tularemia Vaccine (LTV) based on *Francisella tularensis* 15 strain increased survival rate of guinea pigs to 1,5 fold in comparison to control groups were reported (ILYUKHIN et al. 2004). Other trails like live attenuated plague vaccine of *Y. pestis* EV76 derivative of NIIEG line (LPEV), or live attenuated vaccine against tuberculosis (BCG) did not show protection. Mice immunized with vaccine against salmonellosis showed an increased survival rate of 2,4 fold longer in comparison to control groups against *B*. *pseudomallei* infection, but less protection in animals tested when challenged subcutaneously with *B. mallei* C-5 strain (MANEZENYUK et al. 1999, ILYUKHIN. 1999). These results also showed that LTV expressed a better immunostimulating effect against the infection with virulent strain of *B. mallei* C-5, in golden hamster (TIKHONOV et al. 1995, VASILIEV et al. 1993). An efficient protection was also achieved after immunizing with tuberculosis BCG, tularemia LTV vaccines and a vaccine based on B*. cepacia*, when challenged with the virulent strain of *B. pseudomallei* C-141 to outbred white rats and mice (TIKHONOV. 1995). Yet, these are solo experimental studies published, which makes the issue of cross-over vaccine difficult to endorse further, as another possible option of vaccine development against pathogenic Burkholderia.

Although different species of animals used in experiments, which were essentially differing by their sensibility to melioidosis and glanders responses vary, some of the heterologous vaccines were able to express immunostimulating effects, but their levels of protection indicated are very low, particularly when the pathogens are introduced to the respiratory systems, the most plausible route of dissemination in case of bioterrorism. As it was showed that white mice (moderately resistant to melioidosis) which were immunized twice with a virulent strain of *B. mallei* C-5, in a range of $1x10^6$ to $3x10^{10}$ cfu doses followed by intrapulmonary infection with *B. pseudomallei* C-141 in doses of $1\text{-}3x10^8$ cfu, induced a weak protective response. The survival rate of individual experimental animals were in a range of 17 to 56 % (MANZENYUK et al. 1999). Although, the cross-protection provide by immunization of vaccine based on *B. mallei* against its closely related *B. pseudomallei* in this study is low, it is a possible indication to develop a vaccine against both pathogens based on single pathogen, which can be used as bivalent vaccine, which needs further investigation to enhance the level of protection achieved yet.

The cross-over protections of vaccines of other intracellular pathogens like, live attenuated vaccines of Tularemia, BCG vaccine of Mycobacterium and the live plague vaccine of Yersinia EV76 line NEIIG were studied in mice and guinea pigs as well as vaccine based another similar study by ILYUKHIN et al. (1999) indicated that BCG vaccine offered only 10 % of protection for white mice against a dose of 1×10^3 LD_{50} cf s.c. challenged with *B. pseudomallei* C-141 strain [Museum of Live culture of Volgograd Anti-plague Institute]. It did not offer any protection for Golden hamsters challenged with $1x\ 10^2$ LD_{50} cfu of *B. pseudomallei* C-141. The *Y. pestis* EV NIIEG vaccine provided 8 %, 12 % of protection of white mice and guinea pigs, respectively against s.c. infection with $1\ x10^3$ LD_{50} cfu of the virulent strain of *B. pseudomallei* C-141. Similar immunization schedules in mice and

guinea pigs with a higher dose of $10^7$and 10^8 respectively, showed also low protective efficacy in all experimental animals.

Although much is known about the epidemiology, clinical manifestation and the courses of melioidosis and glanders, and to some extent their responses to antimicrobial agents, the knowledge of the host immune system towards the infections are just at the beginning. In order to understand the mechanism of the immune response during infection with *B. pseudomallei* CHENTHAMARKSHAN et al. (2001) investigated sera collected from septicemic patients and healthy groups indicated the presence of high levels of IgG and IgM in 85-100 % of the cases as compared to control groups. Highest antibody response was to IgG and lowest for IgM. IgG1 and IgG2 were the predominant subclasses produced against culture filtrate antigen of *B. pseudomallei* reflecting preferential responses to protein and carbohydrate epitopes.That indicates efficient vaccine must induce IgG subclasses.

4.2.4 DNA /Subunit/Recombinant Vaccines

The live vaccine strain of Tularemia, *F. tularenisis* 15, transformed to R-form that carried two DNA fragments with a size of 4.0 mD and 2 mD isolated from *B. pseudomallei* C-141 constucted by plasmid (pRM2) was studied as vaccine against virulent *B. pseudomallei* strains (ILYUKHIN et al. 1999, 2002,2004, MANEZENYUK). Experimental animals were challenged subcutaneously with typical virulent strains of *B. pseudomallei* -C141, *B. pseudomallei* -57576, *B. pseudomallei*- 100, using a doses of $1x10^2$ LD_{50} cfu for golden hamster and $1x10^3$ LD_{50} cfu for other experimental animals like guinea pigs, white rats and white mice. Immunization was carried out with *F. tularensis* 15 (NIIEG Culture collection) and *F. tularensis* 15 RM2(tab 6) The survival rate of guinea pigs and rats immunized with *F. tularensis* RM2 recombinant vaccine challenged with the virulent strains of *B. pseudomallei* (tab. 4) increased the resistance of animals from 15 to 40 % except the Syrian golden hamster (ILYUKHIN et al. 1999, 2004). White rats and mice did survive slightly longer than golden hamsters, that indicates the vaccine provide no significant protection. Although tularemia vaccine strain was proposed as a potential vector in investigating the protective antigens of intracellualr pathogens for in recombinant antigens synthesis , it was assumed that the drawback of of *F. tularensis* as a vector for the production of recombinant vaccines against melioidosis and other recombinant vaccines is, that they are difficult to reproduce, instable and the inserted section of the targeted vaccine is not known and there are also concerns of safety and the high virulence of the vector itself.

Table 6: Protection eficacy of *rF. tularensis* RM2 and *F. tularensis* 15 immunization against virulent strains of *B. pseudomallei* against s.c. Infection

Immunization	Infection	Animals	MTD	Survivors/total	% Protection
F. t. RM2	C-141	Golden hamster	1	0/5	0
F. t.RM2	C-141	Guinea pigs	4	2/12	15
F. t. RM2	100	Mice (CBA)	4	2/9	22
F. t. 15	C-141	Mice (CBA)	3	4/18	22
F.t.15	100	White rats	5	9/20	40

Notes. F.t. RM2- *F. tularensis* RM2, F.t 15-*F. tularensis* 15. C-141-B. *pseudomallei* C-141, MTD- Mean time to death in (days), compiled from Ilyukhin et al. 1999, 2004.

The majority of recent studies concentrate on DNA-based vaccine strategies of selected isolates of immuogenic antigens as a novel approach for immunoprophylaxis against glanders and melioidosis. DNA vaccine is supposed to have more advantages as to that of classical types of killed or attenuated vaccines in that, DNA vaccine induces cellular immunity, which is indeed the most essential part of the immune system to protect and clear up disease cause by the intracellular pathogens like the agents of melioidosis and glanders from the host. The protective immunity results from the endogenous expression of foreign protein by host cells and the antigen is therefore presented to immune system in a manner that it mimicks the natural forms of infection. Those being the advantages of DNA vaccine, a recurring observation in clinical studies reported in in the literature so far are that antibody responses induced by DNA vaccination are relatively low in human as compared to laboratory animal models. In addition high doses are required to induce substantial T-cell immunity (DONNELLY et al. 2003, HANKE. 1999), and studies on safety concerns of DNA based vaccine are at the beginning phase.

The plasmid DNA encoding the flagella protein (flagellin) vaccine was reported in induction of humoral and cellular immune response and provided a protection of Five out six (83%) BALB/mice immunized with flagellin-DNA vaccine against i.v. challenges with 10^5 cfu of *B. pseudomallei* was confirmed (CHEN. 2006). It was also shown that structural gene, artificially attenuated strains including *B. pseudomallei bipD* mutant, provided 50 % protection against challenge with wild type infection (STEVENS et al. 2004).

Evaluation of the immunogenicity of selected proteins (*LolC*, *PotF*, and *OppA*) of the ATP-binding cassette systems of *B. pseudomallei* as a candidate vaccine antigen where non membrane regions of the *B. pseudomallei* proteins were expressed and purified from *E. coli* and then evaluated as vaccine candidates in an established mouse model of *B. pseudomallei* infection showed that the proteins were able to stimulate antigen-specific humoral and cellular immune responses. Immunization with *LolC* or *PotF* protein domains afforded significant protection against a subsequent challenge

with *B. pseudomallei*. The most promising vaccine candidate, LolC, provided a greater level of protection when it was administered with immune-stimulating complexes complexed with cytosine-guanine rich (CpG) oligodeoxynucleotide 10103. Immunization with LolC also protected against a subsequent challenge with a heterologous strain *of B. pseudomallei*, demonstrating the potential utility of this protein as a vaccine antigen for melioidosis (HARALAND et al. 2007). If it has the some effect against the causative agent of glanders is not known yet.

B. pseudomallei is a resilient bacteria that can survive in variety of hostile conditions, Like many saprophytic organisms, including nutrient deficiency, acid and alkali pH disinfectant and antiseptic solutions, exposure to many antibiotics and extremes temperature. Whereas its closely related facultative intracelluar *B. mallei* is an obligate pathogens that does not exist in nature (WAAG and DESHAZER 2005, CHENG and CURRIE 2005), whose natural hosts are horse, donkeys and mules. However, many animal species ca be infected naturally including carnivores, like line and dogs eating infected meats (ALIBASOGUL et al. 1986). Indicating that both pathogens are well adapted to their many hosts and expressing an impressive array of both, secreted and cell-associated antigens, in order to adhere, invade, colonize, grow and develop a specific infectious process and combat the immune system of the host, able to survive and reproduce in the host cell. Direct cell-to-cell spread has also been observed in human tissue, which was thought to occur by the induction of cellular protrusion and fusion of cell membranes and forms multinucleated giant cells (BRETT and WOODS 1996, BRYAN et al. 1994, CHENG and CURRIE 2005*).* The production of glycocalyx polysaccharide capsule by pathogenic *Burkholderia* species is probably thought to play an important virulence factor in protecting the microbe from unfavourable environment, host defense mechanisms and resistance to antibiotics (STEINMETZ et al. 1995).
The completed genome of *B. mallei* ATCC23344 revealed putative gene products that were similar to virulence factors in other Gram-negative organisms, cell associated as well as secreted, including a type II, type III secretion systems, type IV pilli might have similar roles, which needs to be elucidated by creating defined mutation and examining their relative virulence in animal models of infection (FINLAY and FALKOW 1997, ZHU et al. 2002). Some of the studies showed that mutants in the general secretory pathway, resulting in failure to secrete protease, lipase or lecithinase, do not appear to result in a reduction of virulence in animal model (BRETT and WOODS, 2000), indicating that exotoxins don't play a significant role in determining the outcome of the organism (WHITE. 2003), in contrast to, several studies in the 1950s reported that culture supernatant fluid of *B. pseudomallei* was toxic for both mice and hamsters (COLLING et al. 1958) and the level of production of toxin was strain-dependent (NIGG et al. 1955).

A variety of animals have been used in experimental models to verify the mechanisms of virulence including inbred mouse strains (VELJANOV et al. 1996) chickens (VESSELINOVA et al. 1996) rats and guinea pigs (ILYUKHIN. 2004) and hamster (BRETT and WOODS 2000), which, showed different results. Thus, an attempt to identify virulence factors may be complicated by the fact the syndrome might be dependent on the strains studied and the choice of animal model and translation of the results to human. Although there are some conflicting results of innate immunity, the role of phagocytes, while the pathogen can survive and multiply within professional

macrophages, including, macrophage /monocytes and nuterophil cells, the induction of both arms of immune system is required for protection.

4.2.5 Immunogenic Antigens against *B. mallei* and *B. pseudomallei*

ILYUKHIN et al. (1997) studied, the level of protection of macromolecular fractions extracts obtained from broth cultures of Burkhoderia species that causes glanders and melioidosis, with less toxicity were examined. Based on the methods of extraction, such liquid forms or acetone dried extracts the Authors enumerated the fragments alpha- numeric such as as fragment C, fragment C1, fragment D, fragment H (AVOROROVA et al. 2004), fragment A, fragment D, fragment E, antigen fragment (Ag8) (KALACHEV et al. 1997, PIVEN. 1991). In addition he also studied some other components extracted from cellwall, cytoplasm, ribosome and murine-protein-complex.

The author found that immunization with some of the combatants of the fractions induced the immune response and as a result the vaccine increased the resistance of the animals form 25-60 % against challenges of infection with virulent *B. pseudomallei* strain in mice (PIVEN et al. 2001).
One of the macromolecular fraction that demonstrated singnificant protection was known as antigen 8 (Ag8)an extract from the outer membrane of both species, It is also as known as one of the virulence factor of the pathogens (PIVEN. 1996). Ag8 is glycol-lipoprotein that consists of 70 % carbohydrate, 16 % protein and 14 % lipid, indicating, that the characteristics of biochemical composition of Ag8 can be considered as a good macromolecule of immunogenic properties. Experimental inoculation of laboratory animals with Ag8-mutant was reported in significant increases of the LD_{50} level and remarkably prolonged the dates of survival (PIVEN. 1991). That indicates Ag8 is capable of exhibiting protection.

In addition to Ag8, investigations of various series of common antigens isolated from the pathogens, using different biochemical and immunological methods and then recombined such as, three fragments designated A, D and E showed better protection as alone and significantly induced cellular immunity (KALACHEV et al. 1997). Moreover in combined form with Ag 8 fragment (A+ D+E+Ag8), demonstrated a better protection in comparison to single or partial combinations. It was reported that, immunization with four recombined fragments, increased the survival rate of immunized mice from 40-90 % when challenged with a dose of 30-100 LD_{50}. And 66 % of rats survived against an infection with virulent strain of *B. pseudomallei* of 13 LD_{50}. Golden hamster immunized with recombinant antigens composed of another fragment know as D, E and F plus Ag8 (D+E+F+Ag8) provided a protection of 40-50 % of against an infection with virulent *B. mallei* strain of 100 LD_{50} (PIVEN. 1991). Further reclassified extracts of immunogenic properties were also known in having protective substances. For instance the combination of three fractions, such as A9, D17 and E13 was also known in inducing protective immunity in white rats against an infection of highly virulent strains of *B. pseudomallei* (KALACHEV et al. 1997). At the mean time it was also reported that there are some know fragments purified such as B4 and B17 that showed immunosuppressive effects. A combination of three fragments (A9+D17+E13) was found to provide more pronounced protection against both causative agents and enhanced activation of the

cellular (T-cells) immune system, that plays a significant role in protection. Therefore, the recombinant fragments were proposed to be considered as a prototype of vaccine based on non-cellualr components of the the cell. Synthetic vaccines based on immunogenic molecules as a vaccine against pathogenic *Burkholderia* species might overcome the drawbacks of traditional vaccine based on killed or attenuated vaccine, in concerns of safety, manufacture and reproduction.

Another investigation of the immunogenic properties of the non-cellular virulence factors, like enzymes, was also made. The three forms of the exoproteases extracted from *Burkholderia mallei* and *B. pseudomallei* were designated as Protease I, II and III, showed that Protease I and III enhanced the survival rate of experimental animals against melioidosis to 2 – 5 folds i.e. 4 folds in comparison to control groups (PIVEN and ILUIKHIN 2000).

A special attention was also given to the porine proteins of the membrane of *Burkholderia* to be used as vaccine. Immunization with a porine membrane protein protected 83 % of golden hamsters against a challenge of 20-50 Dcl (where 1Dcl≈ 100 cfu) virulent strain of *B. mallei* (SUPOTNIKII et al. 1993). In order to achieve a better synthetic peptide vaccine it is of value to consider antigen 8 (Ag8) protease I, III and porine protein (ZHUKOV et al. 1997).

Clinical testing of a synthetic capsule-polysaccharide based vaccine against *H. influenzae* type B or Hib, was recently licenced for clinical use, a major cause of invasive bacterial infection, meningitis in children (VEREZ-BENCOMO. 2004). Native Hib capsule was shown to be protective in adults but was poorly immunogenic during infancy and requires the conjugation of Hib capsule to a protein carrier such cholera toxin or tetanus toxoid to provide protection (JACOB et al. 1985, SCIENCE. 2004). Synthetic vaccines have the main advantages in production: higher quality, purer, affordable costs and safety. It will be the most realistic approach in developing vaccines for humans as opposed to a viable organism. They have been shown to elicit neutralizing antibodies in some viral infections of animals like FMD, Rabies and certain animals pathogen (MURPHY et al. 1999) and some human bacterial pathogens, but in general this approach raises more questions, because peptides are linear aminoacids, lack any forms of conformation und could not induce both arms of the immunesystem which is required for protection against infections caused by pathogenic Burkholderia species.

4.2.5.1 Secretory and Cell associated Antigens

B. pseudomallei produces several secretory and cell-associated antigens including, mucous endotoxin, exotoxin, protease, lecithinase, lipase, phosopholipase C (PLC), hemolysins, siderophores, and series of biopolymers (ASHDOWN et al. 1990, PIVEN and ILYUKHIN 2000, LEE and LIU, 2000, WAAG and DESHAZER 2005, COLLING et al. 1958).

Studies conducted by SEXTON et al. (1994) have confirmed the presence of a 36 kDa antigen associated with protoelytic activities in *B. pseudomallei* culture supernatants. In particular, a protease expressed by *B. pseudomallei* 319a was found to be metalloenzyme requiring iron from maximal protease activity and demonstrated optimally activity at pH 8.0 and 60°C. GAUTHIER, et al. (2000) suggested that there is no correlation between virulence and the level of exoproteolytic activity of *B. pseudomallei* strain studied.

Numerous gram-negative bacteria, including both animal and plant pathogens, encode specialized type III secretion system (T3SS), that deliver bacterial proteins (effector molecules) into the plasmamembrane and cytoplasm of eukaryotic cells (HUECK. 1998). Several investigations identified T3SS in *B. pseudomalli,* and *B. mallei,* that T3SS is found to be crucial for pathogenesis (RAINBOW et al. 2002, WINSTANLEY and HART, 2000). In *B. pseudomallei* mutation of the gene (*bsaZ*) showed a drastic reduction in intracellular replication within J774.2 murine macrophages, failure of endocytic vacuole escape and inhibition of actin polymerization (STEVENS et al. 2002, 2004). This suggests T3SS plays an important role in *B. pseudomallei* virulence in all of its animal host (WARAWA and WOODS 2005). Virulence related investigation done on *B. mallei* T3SS, in two species of experimental animals i.e., Syrian hamster and the murine model showed that T3SS mutants were attenuated in hamsters model to LD_{50} values of greater than 10^6 cfu (the wild-type LD_{50} was less than13 cfu) (ULRICH and DESHAZER 2004). This indicates that *B. mallei* is much more dependent upon the T3SS for its virulence in animals than *B. pseudomallei* (WARAWA and WOODS. 2005). Its role as protective antigen is not yet elucidated, however, vaccines based on mutants failed to elicit a protective immunity in BALB/C mouse and Syrian hamsters (ULRICH and DESHAZER 2004), that indicates T3SS possesses immunogenic characteristics. Further investigations are needed to determine the level of T3SS protection.

Genomic analysis of *B. mallei*, indicates that genes encoding an exopolysaccharide capsule (EPS), a lipopolysaccharide (LPS), type IV pili, as well as type III and type VI secretion systems are associated with virulence of the pathogen (WHITLOCK et al. 2007). Although *B. mallei* contains less DNA than genomic comparison revealed significant homology, being ca. 99 % identical between conserved genes of *B. pseudomallei* (GODOY et al. 2003, NIERMAN et al. 2004). That indicates the potent of developing vaccine based on common protective antigens against both pathogenic *Burkholderia* species.

Cell-associated antigens include lipopolysaccharide (LPS), capsular polysaccharides (CPS), flagellin protein (BRETT and WOODS 1997, STEINMETZ. 2000). A number of cell-associated antigens have been demonstrated to be immunogenic in patients with melioidosis including CPS, LPS and flagellin protein (CHARUCHAIMONTRI et al. 1999, DESHAZER et al. 1998) and have been proposed to be used as vaccine components and serological diagnostic. Capsule from gram-negative and gram-positive bacteria are also known to be important virulence factors in the pathogenesis of invasive diseases (MOXON and KROLL 1990) because of their ability to protect the bacteria from the host defense system. They may play an antiphagocytic role, as they may induce cytokines for example TNF, IL8 and IL10 in various leukocytes population (GIBSON et al. 1996, POWELL et al. 1997) and thereby, influence the type of immune response elicited by the host. Outer membrane proteins, such as protein tyrosine phosphatase, have been defined to play a role in invasion and virulence (KONDO et al. 1991), although *Burkholderia* do not contain substrates tyrosine phosphate, analogous enzymes in other bacteria such as *Yersinia* have alternative substrates that are believed to be important in signal transduction (KANAI and KONDO. 1994). However, acid phosphatase do not appear to be a major virulence determinate factor as strains with mutations of *acpA*, resulting in lose of phosphatase activity, retains their virulence (BURTNICK et al. 2001).

4.2.5.1.1 Capsule

Capsule production has been correlated with the virulence in many bacteria, particularly those causing serious invasive infections in humans (BOULNOIS and ROBERTS 1987, BOULNOIS. 1989). A number of functions have been suggested for polysaccharide capsules: prevention of desiccation for transmission and survival (KANAI and KONDO 1994) adherence for colonization, resistance to complement mediated phagocytosis and complement-mediated killing, and resistance to specific host immunity (PUTHUCH-EARY et al. 1996) due to a poor antibody response to the capsule Poly-saccharide. Capsules are highly hydrated polymers that mediate the interaction of bacteria with their immediate surrounding (ROBERTS 1996).

B. *mallei* was known as non capsule producer in old literature, on the other hand numerous studies prove that *B. mallei* does make a capsule, that is important for virulence (POPOVet al. 1991, 1995, 2000, RECKSEIDLER et al. 2001, DESHAZER et al. 2001) Thus, the capsule of *Burkhlderia* species is suggested to protect in early the pathogen from being phagocytized and may block the microbicidal action of phagocytes after internalization (RECKSEILDER et al. 2001, POPOV et al. 1991, 2000). It is possible the capsule may confers resistance of the bacteria to the hosts primary response to escape and able to replicate intracellular.

A survey of phenotypic traits that are present in *B. mallei* and *B. pseudomallei*, but absent in *B. thailandenssis*, may allow the identification of new virulent determinants. A capsule-like exopolysaccharide is one of the candidates that fit these criteria. The exoploysaachride (EPS) produced by *B. pseudomallei* is an unbranched polymer of repeating tetra-saccharide units with the biochemical structure of•3)-2-*O*-acytel-β-D-Gla*p*-(1-4)-α-D-Gal*p*-(*1-3*)-β-D-Gla*p*-(1-5)-β-D-KDO*p*-(2-(NIMTZ et al. 1997, MASOUD et al. 1997). The genes encoding the EPS of both pathogens have

not been identified, and its role in pathogensis is currently unknown (WAAG and DESHAZER 2005), but antibodies against EPS have been shown from melioidosis patient's sera (STEINMETZ et al. 1995), that indicates EPS exhibits immunogenic properties, to be include in developing vaccine against Glanders and Melioidosis.

The capsular polysaccharide of *B. mallei* is required for production of disease that was observed in in two animal models (hamsters and mice) of glanders infection and is a major virulence factor. Surprisingly the non-pathogenic *Burkholderia thailandensis* also harbores a portion of the capsular gene cluster (DESHAZER et al. 2001). The differences of virulent CPS of pathogenic strains versus non pathogenic strains of Burkholderia are not yet elucidated.

4.2.5.1.2 Lipopolysacchride

The LPS of *B. pseudomallei* has been reported to contain two types of O-poly-saccharide moieties termed type I O-PS (unbranched homopolymer of mannohepto-pyranosyl residues) and type II O-PS (unbranched heteropolymer) (KNIREl et al. 1992, PERRY et al. 1995) which has been shown to be involved in serum resistance (DESHAZER et al. 1998). Mutants lacking type II O-PS were found to be sensitive to bactericidal activity of 30 % normal human serum (NHS) and also demonstrated reduced virulence in three animal models of infection. Thus, the type II O-PS is essential for *B. pseudomall*ei serum resistance and virulence. Similarly, *B. mallei* strains that are deficient in LPS *O*-antigen are killed by 30 % NHS (DESHAZER et al. 1998, BURTNICK et al. 2001)

In previous studies, it was suggested that structure of LPS of *B. mallei* can be similar, possibly identical to *B. pseudomallei*. It was suggested, that it consists of a protein with molecular mass of 20-60 kDa, a glucoprotein of 90-200 kDa and a lipoprotein with a molecular weight of 18-20 kDa (POPOV et al. 1991, 1995, 1997). The capsule formed by *B. mallei* was shown to be one of the factors facilitating its persistence in the body of the host (POPOV et al. 2000). Now there is a suggestion that describes the structure of *B. mallei* LPS, is as -3)-β-D-glucopyranose-(1,3)-6-deox-α-L-talopyranose- (1- in which the talose residue contains 2-*O*-methyl or 2-O- acetyl substituent (BURTNICK et al. 2002, WAAG and DESHAZER 2005). *B. mallei* LPS *O*-antigens cross-react with polyclonal antibodies raised against *B*. *pseudomallei* LPS *O*-antigen (DESHAZER et al. 2001, BURTNICK et al. 2002).

B. pseudomallei strains isolated from a variety of sources have homogeneous, highly conserved and constant LPS. LPS is a component of the outer cell membrane of gram-negative bacteria that is an important factor mediating the production and release of various mediators (such as NO) and cytokines from microphages through CD4. *B. pseudomallei* invaded and multiplied inside macrophages without substantial macrophage activation. The inducible nitric oxide synthase (iNOS) expression was not observed even when the cells were infected with *B. pseudomallei* at high multiplicity of infection (MOI). However, during these time intervals the macrophages did not produce sufficient quantity of nitricoxide (NO) to be detected in the supernatant by the Griess

method. Unlike *B. pseudomallei,* the cells infected with other gram-negative bacteria, such as *E. col*, or *S. typhi*, were able to express iNOS and released higher quantity of TNF-α at considerably lower MOI ratio (PONGSAK et al. 2001). These findings suggest that *B. pseudomallei* exhibits a unique mechanism for intracellular survival, most likely by interfering with normal cellular defense. Thus, the unique characteristic of both pathogens, their unusual structure of LPS, may facilitate its survival inside the cells which may in turn allow the bacteria to have a long latency state in the host.

The O-PS side-chains of LPS are integral serum resistance determinants in some gram-negative bacteria (TAYLOR. 1983, JOINER. 1988). Performance of ELISA and mono-clonal antibody (mAb) specific for type II O-PS showed that serum-sensitive mutants lacking type II O-PS did not react with specific mAbs. That suggests a correlation between serum resistance and the presence of type II O-PS (BRAYN et al. 1994, DESHAZER et al. 1998).

Comparative studies made between a serum-sensitive mutant named SRM117 and its parental strain 1026b in three animal models of *B. pseudomallei* infection, Golden hamster, (*Mesocricetus auratus*), Guinea pigs and infant diabetic rats showed, that the 50 % lethal doses (LD_{50}s) for 1026b and the SRM117 in the hamster model of infection were <5 and 62 and in the guinea pig 2 x 10^3 and 2 x 10^4, respectively. In case of the infant diabetic rat the LD_{50} s for 1026b and the SRM117 were 2 x 10^4 cfu and >5 x 10^6cfu, respectively. Thus, the serum-sensitive mutant was approximately 10-fold less virulent than the parental strain 1026b in the hamster and guinea-pig and was more than 100-fold less virulent in infant diabetic rats model of melioidosis (DESHAZER et al. 1998, WAAG and DESHAZER et al. 2005, CHENG and CURRIE 2005). That also suggest the type II O-PS moiety of LPS is an important virulence determinant factor for pathogenic *Burkholderia* species. In recent publication the term LPS is used more often as II O-PS. *B. mallei* candidate conjugate vaccines, linking to the capsule and O-PS lipoploysaccharide-flagellin antigen were also demonstrated protecting of infant diabetic rats in passive immunization (BRETT. 2000), however, the conjugates are not tested in active immunization.

4.2.5.1.3 Flagella, Siderophore, and Pilli

Flagella are commonly recognized as important virulence determinants expressed by bacterial pathogens since the motile phenotype imparted by these organelles often correlates with the ability of an organism to cause disease (PENN and LUKE 1992, MOENS and VANDERLEYDEN 1996). Flagella are filamentous extracellular appendages that are employed by bacteria to move toward environments that promote their survival (WILSON and BEVERIDGE 1993) and are composed of 3 main elements: the flagellar cork-screw like propeller (filament), the universal joint (hook), and the transmission shaft, motor and bushing (basal body) which is embedded in the cell envelope. Flagellar filaments consist of one or two repeating subunits of identical polypeptide monomers called flagellin (HAKALEHTO et al. 1997).

The flagella and motility, as well as the resistance of the organism to the bactericidal action of normal human serum, are believed to facilitate the dissemination from sites of localized infection, such as the lungs or skin, to virtually any other organ of the body via the blood circulatory system (DESHAZER et al. 1997, ISMAIL et al. 1998). In several bacterial pathogens such as *S. enterica* serovar Typhymurium and *Vibrio cholerae*, virulence has been correlated with flagella and bacterial motility, but it is not clear how flagella function as virulence factors (CARSIOTIS et al. 1984, GARDEL and MEKALANOS 1996). Flagellar motility has been reported to enhance the invasion of the host cells by *Campylobacter jejuni, Proteus mirabilis, Vibrio anguillarum,* and other pathogenic species (MOBLEY et al. 1996, ORMONDE et al. 2000, YAO. 1994), whose role is attributed to the prospects of the filaments carrying adhesions for attachment to the intestinal mucosa, furthermore, may also assist bacteria in nutrient and waste exchange (WILSON and BEVERIDGE 1993). For example, a nonmotile mutant of *B. cepacia* with a defective component of the motor-switch complex of the flagellar basal body was found to be less invasive in lung epithelial cells. Flagellum-mediated motility has been implicated in the pathogenesis of *B. cepacia* because it facilitates penetration of the host epithelial cell barriers and contributes to the onset of systemic spread of the organism (TOMICH et al. 2002).

Several genes are involved in the motility. They have been identified by transposon mutagenesis of *B. pesudomallei*. Among these is *fliC*, which encodes flagellin (DESHAZER. 1997). Antibodies rose against the *B. pseudomallei* flagellin markedly reduced the motility of the bacterium and provided passive protection against *B. pseudomallei* infection in animal models (BRETT et al. 1994). CHUA et al. (2003) found that the flagellum is an important virulent determinant factor in the pathogensis of *B. pseudomallei* based on the mouse model. In contrast, DESHAZER et al. (1997), showed that there were no significant difference between the virulence of the wild type and the virulence of the Tn5-OT182 disrupted *fliC* mutant when the pathogens were injected intraperitoneally into young diabetes rates and Syrian hamsters.The difference in the findings could have been due to the use of different animal models since the disease in diabetic rats and hamsters was more acute than in mice.

The causative agent of melioidosis, *B. pseudomallei* a motile gram-negative bacillus, that moves by means of polar tuft of two to four filamentous flagella, in comparison to the constructed non-motile mutant from virulent strain isolates of *B. pseudomallei* that lacks the flagellin (*fliC*) could also invade cells as effective as the wild-type bacteria, and showed no attenuation in virulence when tested on *Caenorhabditis elegans* as an alternative host. However, infection of mice through the intranasal route with the mutant did not cause disease whereas wild-type bacteria killed the mice within two weeks. Therefore, although flagella is not necessary for bacteria to invade cells in vitro and to infect *C. elgans*, it is important in the pathogenesis of *B. pseudomallei* in the mucosal infection of mice (FINKELSTEIN et al. 2000, CHENG and CURRIE 2005, GAN et al. 2001).

The very closely related *B. mallei,* causative agent of glanders, is amotile. Surprisingly genes which are responsible for filament-forming flagellin *(fliC*) are found in *B. mallei*, where the only identified alteration of this gene in comparison to the motile *B. pseudomallei* is a shift from G to C at gene position 798 in all 7 *B. mallei* strains investigated (SPRAGUE et al. 2002). The reason why *B. mallei* lacks flagella remains obscure.

Bacterial flagellin has been recognized as being an immuno-stimulator, capable of activating NF-kB-signalling and subsequently induces the secretion of inflammatory mediators such as NO, TNF-α, IL-1, IL-6 and IFN-γ, factors which amplify the overall immune performance of the host after an infection with flagellated bacteria (RUCKDESCHEL et al. 1998, EAVES-PYES et al. 2001, MCDERMOTT et al. 2000). Flagellin is also believed to present T-cell epitopes on antigen-presenting cells (APC), and to induce maturation of APC, resulting in an expansion in the number of naive T cells (MCSORELY et al. 2002). These finding indicate that flagellin is an immunogenic substance that can be considered as a good candidate vaccine to be included in vaccine development against melioidosis. It is not known if flagellin will have the same effect against glanders.

A conjugate molecule incorporating both, flagellin protein and O-polysaccharide antigens were proposed to be a reasonable vaccine candidate for use in active immunization against melioidosis, because both antigens have shown a high degree of conservation on structural and immunological levels. It is present in the majority of strains tested so far (BRETT et al. 1994, BRYAN. 1994, KNIREL et al. 1992, PERRY et al. 1995). Thus, a conjugate molecule incorporating both flagellin and the PS antigens would be a reasonable vaccine candidate for the use in active immunization against melioidosis and glanders. Flagellin gens are also proposed to be used as biomarker for detection of population genetics and epidemiologicaly analysis (WINSTANELY. 1997). An increase in IgG titers to LPS of strain 319a and flagellin protein was clearly demonstrated in the serum of vaccines after primary as well as two subsequent booster doses in rabbits (BRETT and WOODS 1996). Rabbit polyclonal antiserum raised against the glycoconjugate preparations was shown to react strongly with both proteins and is both, functional and specific (BRETT et al.1994). The purified immunoglobulin fraction utilized for passive immuno-protective studies in the animal model of diabetic rats showed that *B. pseudomallei* infection with LD_{50} of 8.89×10^5 for immunized group

and 7.54 x 10^3 for control group showed, that immunized groups displayed a 60 % survival rate over the same time; where as only 20 % of the animals of the control group were still alive after the eighth day of challenges (BRETT and WOODS 1996, BRYAN. 1994).
The utilization of iron i.e. the ability to acquire iron from host sources is the prerequisite for the successful establishment and maintenance of most bacterial infections. YANG et al. (1991) have demonstrated that all 84 strains of *B. pseudomallei* examined during their studies tested positive for siderophore production. Further investigation to illustrate the structural and chemical analysis of the siderophore synthesis by *B. pseudmallei* U7 strain confirmed that the molecule was approximately 1,000 Da in size, water soluble with a yellow-green flourescence and that it belonged to the hydroxamate class and the name malleobactin has been proposed for this compound. Furthermore studies have also demonstrated the siderophore was capable of scavenging iron from both, lactoferrin and transferrin, *in vitro* (YANG et al. 1993).

To asses the role of *B*. *pseudomallei* siderophore production in determining the level of virulence of the bacterium isolated from clinical or environmental samples studied by ULETT et al. (2001) in terms of virulence in BALB/c mice demonstrated that the level of virulence of *B. pseudomallei* in mice does not correlate with the level of siderophore production, although the production of siderophore was determined subsequently by each strains relatively. There was also no significant difference between the levels of siderophore production, by clinical as opposed to environmental *B. pesudomallei* strains. These results collectively indicate that siderophore production plays a limited role in the virulence of the pathogen. A high of the *B. pseudomallei* variability and within species lead to strong difficult in identification of the pathogen virulence as well as the immunogenic factors.

Other cell-associated antigens include type I pili (encoded by *fimA*, *fimC* and *fimD*) and a putative type IV pilus are also mediated motility and pili are also known as virulent determinants of *B. pseudomallei* and it is proposed to be a protective antigen against the pathogen, but it is not clear how flagella and pilli functions as virulent factor and what level of protection it provides against melioidosis and glanders (CHEN. 2006).

4.2.5.1.4 Antigen 8

Antigen 8 is a glycoprotein of 289 kDa molecular weight, an extracelluar capsule–like substance found on the surface of both pathogenic Burkholderia species (PIVEN et al. 1996, SAMYGIN et al. 2001, KHRAPOV et al. 1998, PIVEN. 2001). The scheme of the *Burkholderia* antigens have been porposed by Russian scientists on the bases of immuno-electrophoretic studies of aqueous-saline extracts from acetone-dried microbial cells belonging to 29 *B. pseudomallei* strains. The mixture consisted of 19 precipitates, whereby 13 of these showed to be common to all strains investigated, the rest been variable. The author designated the antigens alpha-numerically i.e. antigen A to antigen K and antigen 1 to 8 (PIVEN and ILYUKHIN 1981). Antigen 8 has an adhesive properties, supposed to be associated with surface capsule-like structure, which is linked with pronounced antiphagocytic and immuno-suppressive function that contributes to survival and maintenance of

the organisms in host cells (PIVEN et al. 1991, BROWN et al. 2002). One of its adhesive properties consists of the hydrophobic characteristic of its outer membrane, which in certain steps is required for the interaction of the bacterial cell with its environment (KAPLIEV. 1990). A comparative study of the adhesive properties of Ag8 of wild type virulent *B. pseudomallei* strain and its mutant defect of Ag8 showed a 34 % reduced adhesive activity in trachial epithelium (PIVEN and ILYUKHIN 2000). This indicates that Ag 8 consists of adhesive properties comparable to that of fimbriae and pilli. Expression of Ag8 correlates with high virulence of *B. pseudomallei* and its absence for decreased virulence. This was confirmed by investigations of biological properties of subcultures which are unable to synthesis Ag8 isolated from highly virulent strains found in Asia and Australia, that showed the virulence of Ag 8^- strains was reduced to 2 $x10^4$ and 1 $x10^5$ foldes, in white mice and golden hamster respectively (PIVEN et al. 1991).

B. pseudomallei can persist in a dormant stage in macrophages for months or years and it has been found to survive and multiply in human phagocytes *in vitro* (HARELY et al. 1998, PRUKSACHARTVUTHI et al. 1990). The mechanism by which this organism survives within human phagocytes is still not fully known. It is assumed that *B. pseudomallei* might make use of host cell components, specifically micro-filaments, to invade eukaryotic cells as other invasive organisms like *Yersina*, *Salmonella, Shigella* do (PERRY and FETHERSTON 1997, MASTROENI et al. 2000). Intracellular pathogens have adapted various strategies to evade host defense mechanisms, including invasion of non-professional phagocytes, escape into the cytoplasmic compartment, interfere with reactive oxygen intermediates, inhibition of phagosome-lysosome fusion and phagosomal acidification, and resistance to lysosomal contents and cationic such as defensins (KAUFMANN. 1993). Pathogenic *Burkholderia* species can kill the host cells by inducing apoptosis in both, pahgocytic and nonphagocytic, cells (KESPICHAYAWATTAN et al. 2000).

Inclusion of Ag8 to other non cellular components extracted from Burkholderia showed some extents of protection in experimental animals (ZHUKOV et al. 1997). That indicates Ag8 fraction can provide protection if used as a possible vaccine component of some other immunogenic fractions extracted from the pathogen as non cellualr/synthetic vaccine against Glanders and Melioidosis, that requires further investigations.

5 DISCUSSION

Throughout the cold war, the causative agent of plague (*Y. pestis*), glanders (*B. mallei*) and melioidosis (*B. pseudomallei*) were considered as the main bacterial agents of military relevance and studied in depth in different states worldwide, genetically manipulated to enhance their potency virulence and drug resistance to the level of untreatable infection for instance in FSU (ALIBEKOV. 1999, DOMARADISKIJ. 1998, DOMARADISKI and ORENT, 2001).

The possibility that an aerosolized form of thes agents could be used as a biological weapon, the capacity of dissemination from person-to-person, wide spread natural availability, higher fatality rate in cases of any sort of pulmonary infection, and furthermore the absence of stringent control system, MDR to chemotherapy and lack of prompt diagnostic methods makes them agents of high concerns of not only public health, but also security, social, economical and not less psychological impacts in human (CDC. 1999, KONDRIK et al. 2003, CFSPH. 2007). Thus, they are included in approved lists of of biological agents at global level.

Just 24 days after the tragic events of 11th September 2001, in USA, the confirmed cases of letters sent to politicians and media offices containing deadly inhalational anthrax spore (*Bacillus anthracis*) was an intentional bioterrorism-related act "*inside job"* (CDC. 2001). and a suspected bioweapons scientist committed suicide reported in *Los angeles times* (2008) dictates us to live in world of fear. Currently, one can raise a question, how do the world act in response, if these pathogens are used to harm a human or suppose if this bio-attack was caused by plague agent instead of anthrax which is not a real respiratory disease, at present no body knows? Furthermore we also do not know from where the danger may come.

Plague is an exceptionally virulent, vector-borne zoonotic disease transmitted from rodents, especially rats, through the bites of infected fleas, most often the rat flea, *Xenopsylla cheopsis*. At least over 200 species of mammals serves as reservoir that including rats, squirrels, mice, prairie dogs, gerbils, susliks, voles for the agent of plague, *Y: pestis*, which persists in the environment as the result of a stable and constant rodent-flea infection maintain the cycle in endemic regions worldwide except Australia (PERRY and FETHERSTON 1997, ANISIMOV et al. 2004). There are also reported cases, where plague was transmitted from non-traditionally associated animals like cats and dogs (GAG et al. 2000), or eating contaminated camel meats to human (ARBAJI et al. 2005).

The unique characteristic related to individual pathogens, highlights that arguably, the development of vaccine as the only appropriate steps to combat against such a disease of high calibre. And that the vaccines should be able to protect against pulmonary infection caused either by deliberate released weaponized strains, the most probable way bioterrorism may take, or undelibarte infection (HASSANI. 2004, CDC. 2005).

In the 20th century two classical forms of vaccines were used in immunization of human against plague worldwide. The first widely used plague vaccine was developed by Haffkine in 1897 using a heat-killed old broth culture of *Y. pestis*. administered s.c., (TAYLOR. 1933) It conferred significant protection against bubonic plague but induced severe adverse reactions including high fever in the majority of vaccines (WILLIAMS et al.1980, MEYER. 1974, ECHENBERG. 2001). Moreover, later studies in rodents and nonhuman primates showed that the vaccine was unable to elicit protection against pneumonic plague and do not provide protection against infectious caused by atypical strains (WILLIAMSON et al.1995, 2001).

Varies modified series of a formalin-killed whole-cell vaccine known as USP, based on highly virulent strains like Shasta, Yerka and 195/P were developed in the mid-20th century in the USA. It was mainly used to protect US military personnel against bubonic plague during the Vietnam War (MEYER. 1964, MARSHALL et al 1974) but; it also caused severe adverse reactions and was unable to elicit protection against pneumonic plague. In the year 1999, the corporation stopped production and it is not more commercially available for human immunization (WILLIAMSON. 2001). The demonstration of their low efficacy, high reactogenicty and their remarkably old technologies of killed vaccines preparations are fortunately, 'antiquated' is not necessarily synonymous with 'bad' some of the traditional vaccines are safe and effective as demonstrated by the recent eradication of smallpox and the expected eradication of poliovirus in a few years (WHO. 2005). Indeed, some of the vaccines developed over the past century would not have met the standards of today and the only reason they are still on the market is that they have been used for many years to vaccinate humans and no new or improved vaccine is developed yet. They have been classified as safe and with a high benefit-to-risk ratio as it is in the case of live EV 76 line NIIEG vaccine against plague used in FSU and continued to be used in some members of CIS (FEDEROVA et al. 2007,2008).

In retrospective survey of plague vaccine research publications data available in English language, It was found that the works of MEYER et al. (1974), MARSHALL et al. (1974), CAVANAUGH et al. (1974), as the final depth of research activities during the cold war era. Following two decades consistent research publications are not available, suggesting that it was easily forgotten. Where as, it was in this period that in FSU the novel achievements, what we today know about plague microbe was discovered and published in Russian scientific journals, dissertations and thesis were written, conferences, congresses were held. It was at this period that the API established the "Biopreparte complex", explored the notorious 43 autonomous natural foci which covers about 10% of its territories, isolated highly intraspecifc diverse strains from various sources like soil, rodents, birds, insects, e.t.c., (NARKEVICH. 1991, ANISIMOV et al. 2004). It was also at this period that the API established its own taxonomy of plague microbe, which is currently used in the region and do not known or included in the taxonomy of International Code of Nomenclature of Bacteria (ICNB) (APARIN et al.1987).

It was also in this period that earlier recognitions of the drawbacks of the killed plague vaccines, extacted fraction extracts of plague pathognes led to search and development other methods of

attenuation, such as heating the culture in presence of concentrated saccharose solution. In FSU studies on Live plague vaccine based on the strain of *Y. pestis* EV 76 proposed by Girard and Robic began in 1936 and the most common derivative strain studied in depth and used for human vaccination is the *Y. pestis* EV line NIIEG strain (ANISIMOV et al. 2002, FEODOROVA. et al. 2007,2008). It is the national standard of plague vaccine strain used and manufactured in different forms and routes of administration, including, subcutaneous, cutaneous, inhalation, or intramuscular immunization. However, the highest level of specific immunity against plague was achieved either by scarifying the dermis followed by injection or after vaccination by inhalation (SALYTKOVA and FABICH 1975, ALEEXEVA et al. 1997, FEODEROVA. 2007). Other forms of vaccine preparations were also studied and used for a limited period of time such as a dry bivalent live vaccine called № 1-7 (KZOLOV et al. 1960). The Derivatives of EV 76 strain were the most widely used live palgue vaccine worldwide (GRASSET. 1942, 1946, MEYER, 1970, 1974, FEODEROVA et al. 2007, 2008).

It was also at this period, that an aerosol immunization/inhalation, with dust plague vaccine based on live EV vaccine strain was developed studied in human (ALEXANDROV et al. 1962), that showed no significant post immunization clinical reaction. Field trails or this immunization method was proved to be simple, allowing mass (herd) immunization of the population to be carried out, such as in case of bioterrorism. Topical immunization via the respiratory tract would be highly beneficial against pathogens that case pneumonia as a primary target during lung infection, while it targets the bronchial and lung epithelium that comprises a large surface between host and environment. Aerosol vaccine has an advantage when compared to traditional routes of administrations, it potentially avoids needle injection and allow herd immunization in short period. Aerosol vaccine can be considered as an ideal emergency prevention measure against plague in case of bio-attack, if mass immunization is essential with in short period of time, if the vaccine is able to elicit rapid, robust and long-lasting protective immunity after vaccination and non-reactogenic.

Other form of vaccine known as “chemical plague vaccine” according to the Author, that consists of capsule antigen and main somatic antigens of plague microbe was also tested in human. It was reported to be superior to live plague vaccine when used as booster immunization and proposed to be included in yearly plague vaccine calendar in the region (ALKESEEVA. 1997, DALVADYANTS. 1997, 2005).

Thus based on documents cited in this work, currently, what is known in English language research publications when compared to that of Russian language publications, it is possible to underscore the following major differences in plague microbe research in general and vaccine development in particular:

1) Differences in new vaccine designs and strategies - After two decades of gaps, the renaissance of plague vaccine research activities in Western hemisphere, began in the mid of 90th, based on new molecular technology, like the development of vaccines based on recombination of of selected major immunogenic antigens of *Y. pestis* inserted in to gram negative pathogens such as

transformed *E. coli* (ANDREWS et al. 1996) or *S. typhimurium aroA*$^{-}$ (GARMORY et al. 2003) containing either F1 or V-antigen alone or in recombined form (WELKOS et al. 1995, MOTIN. 1994, LEARY. 1995, HEATH. 1997, 1998, WILLIAMSON. 2001) .The successes of these vaccines were mainly measured in comparision to USP (killed) vaccine. No comparative studies of recombinant antigens (F1+V) or Sub-unit (F1-V) to the live EV vaccine strain, more over of EV 76 line NIIEG, mostly used in CIS, are available either in animal models or human trails. Whereas, in FSU/CIS, the efficiency of various types of vaccines previously studied, and the forthcoming vaccine candidates, some new, some improved were/are measured in comparison to the paternal live plague vaccine of EV 76 line NIIEG (LEBEDENSKII. 1971, ANISIMOV et al. 2002, FEODOROVA et al. 2007).

Plague vaccine studies predominantly concentrates on two major immunogenic proteins, F1, and V-antigen, which are currently, under trail phase, the rF1 + rV (Salsibury, UK) also known as recombinant plague vaccine, and F1-V (USAMRIID, Fort Detrick, USA) known as sub-unit vaccine. Although these vaccines are able to protect mice and non human primates but not guinea pigs from pneumonic plague and plague induced by atypical strains F1- are reported and tolerable in human trails (LEARY et al. 1995, TITIBALL and WILLIAMSOM 2001, WILLIAMSON et al. 2005). LcrV triggers the induction of IL-10 by host immune cells and suppresses proinflamatory cytokines as well as innate defence mechanisms required to combat the pathogenesis of plague which may preclude the use of V-antigen as a human vaccine (NAKAJIMA. 1993). *Y. pestis* can bypass protective antibodies to LcrVand activation with IFN-gamma to survive and induce apoptosis in murine macrophages (NOEL et al. 2009). Vaccines based on selected antigens like F1-V in subunit or recombinant against plague might have a limitation in providing universal protection. It is not know if it can provide a protection against a number of atypical strains, isolated from different regions of the world for instance the highly diversified strains known in the FSU (ANISIMOV et al. 2004) which might be more attractive to be used for intentional purpose in bioterrorism. There are also other emerging groups of Yops which are known to be protective in experimental animal models. Although some experimental studies made based on modified DNA derivatives of known major immunogenic antigens in prospects of new vaccine development against plague are available, they are irregular.

Currently, researches activites of plague in FSU/CIS focuses on the improvements of live attenuated vaccine EV 76 strain line NIIEG. Currently, the new vaccine candidate, the *Y. pestis lpxM*-mutant live vaccine has been know in providing more protection, less toxicity, in three animal models studied and was reported in inducing enhanced immunity against bubonic plague and is under investigation for further development (FEODOROVA et al. 2007, 2008). Additionally a number of vaccine candidates of *Y. pestis* strains, with different contents of plague antigens are under investigation in comparison to live plague vaccine EV76 line NIIEG, to develop an improved live attenuated plague vaccine.

2) National standard of instructions and methods of evaluating new strains for plague vaccine development- The major criteria of evaluating candidate strains of plague vaccine was used for more 7 decades and confirmed by the API experts and authorities like Ministry of Health of Russian Federation and implemented currently for plague vaccine research. These resolution includes the

guidelines and major criteria that a new candidates for new plague vaccine must fulfil i. e.: i) candidate vaccine must able to induce residual virulence in mice infected with higher dose, and in guinea pigs, indicates that the candidate strain must be able to replicate in experimental animals during the first two weeks after infection, ii) candidate must belong to group three security level, iii) must be pgm^- (*pgm* mutant, iv) must harbor 3 plasmid DNA with corresponding molecular mass, pFra (60 MD), pCad (47 MD), and pPst (6 MD), v) candidate strain for plague vaccine production must protect against aerosol and s.c. challenged white mice with virulent strain and must induce efficient protection and sustain for longer period, vi) have typical cultural–morphological characteristics. Another important properties of candidates vaccines immunology, like minimal and lethal doses, level of reactogenicity, methods of determining residual virulence, acceptable cellular alteration, routes of infection and immunization, methods of serological detection of the activities of immunological cells, were exactly predetermined in varies species of experimental animals and must be able to lysis by *Y. pestis* pahge L-413C based diagnosis (MoH. 1970, ANISOMOV. 2002).

3) Limited genotypic and phenotypic strains are known in Western hemisphere - The majority of studies of plague pathogen and plague vaccine, and what is known in the west (English publications) are based on limited *Y. pestis* strains predominantly the *Y. pestis* CO92 (HEATH. 1998, ANDREWS et al. 1996, 1999, ANDERSON. 1996) *Y. pestis* GB (DENG et al. 2002, JONES et al. 2003, WILLIASON. 2002, GRIFFIN et al. 2005), *Y. pestis* KIM (WANG et al. 2004), which are known to be genetically identical to the extent of over 98%. The first known strain in the west isolated from the former Soviet Union is, *Y. pestis* Pestoides F, sequenced by GARICA et al. (2007) which brought the classical methods of classification of plague pathogens used currently in Western hemisphere in question. Unlikely there are members of an atypical group of *Y. pestis* from Central Asia, Russia denominated as *Y. pestis* subspecies caucasica which varies from several classical *Yersinia pestis* strains still not well studied. These strains, are distinguished by a number of characteristics including their ability to ferment rhamnose and melibiose, their lack of the small plasmid encoding the plasminogen activator (pla) and pesticin, and their exceptionally large variants of the virulence plasmid pMT (encoding murine toxin and capsular antigen) and are proved to be virulent. For instance alone from Central Asian desert natural foci region (UralEmba), 6,5 % of the isolate where atypical strains, 9,93 % of them were $F1^-$, 7,14 % were $LcrV^-$ And 1,36 % of them were pst^-. That indicates a high numbers of intraspecific genetic and phenotypic diversity among *Y. pestis* circulating in natural foci found in GUS alone, where consequent opportunity for genetic exchange and rapid growth of new phenotypes are to be seen. Recent observation made also showed that there are also differences between two isolates of the “classical” antiqa biovar, strains Antiqa and *Y. pestis* Nepal516 lineages DNA sequenced recently (CHAIN et al.2006). That indicates availability of high genomic diversity among *Y. pestis* isolates from different sources and regions of the world, that needs to be considered in order to develop a vaccine against plague with universal protection and global implication. The API of Russia possess the most diversified strains collection of plague pathogen in the world which are not yet known outside Russia.

4) Preferences of Animal models in plague research- The most used Animal models in Western hemispheres relay on mice as model in studying plague vaccine for human use, such as Swiss Webster, BALB/C mice, e.t.c., (WORSHAM. 2004). Attaining the ultimate goal of vaccines that are

safe and efficacious in humans can be both advanced and hindered by studies in single animal systems. Guinea pigs are categorized as bad model for plague studies by western experts and are rarely used. In contrast in FSU/ CIS standard plague vaccine studies as a rule of law must includes at list three species of experimental animals with different level of sensitivity i.e. mice, guinea pigs and hamsters initially. Further studies include wild rodents, which are known as carrier in the natural foci, and primates and finally human. It is known that different species of experimental animals respond differently to the plague pathogen in their sensitivity, immunogenicity, and pathogenesis. Thus results of studies do also vary depending on the model animal used, and further more difficulty, when the ultimate goal of plague vaccine development is to protect human, including children and elders, eventually differs in the level of immunological status. Thus, translation of a successful vaccine results based on single species such as in breed or outbreed mice models of animal research might led to less efficient, less acceptance to global level, might not able to provide universal protection against the highly diversified strains known in different regions of the world. It is also crucial to take into consideration, that more than 200 species of animals and insects are known to harbor plague pathogens in natural foci. Thus, studies of plague in multiple animal models is more reasonable than studies based on single species to evaluate the efficacy of plague vaccines because human clinical trials that test new vaccines are not feasible.

There are also a great concerns in regards to vaccine development based on live attenuated microorganism, a concern, that vaccine strain might be able to reverse to virulence, induces infection, that being the explanation why live attenuated EV strain was not licenced against plague in the last century in the west. At the mean time it is also known that vaccines based on live attenuated microoragnisms are superior to all forms of vaccines know today in inducing the appropriate immune responses. The licenced vaccine of EV 76 line NIIEG is known in inducing both arms of the immune system, which are crucial for protection of intracellular pathogens like *Y. pestis.* Unlike other EV 76 strains used as live vaccine worldwide, the derivative of line NEIIEG, showed no signs of revert and proofed to be stable. The humoral and cellular immune systems are both required in order to provide efficient protection against pneumonic plague, which any forms of forthcoming new vaccine must fulfil.

5) Continuity and intensity- Further development of new or improved, live attenuated vaccine against plague in Russian is based on consistent monitoring of the maternal strain line EV NIIEG since 1936 that proofed to be stable, safe in all its physico-biochemical, immunological values. This consistent observation was made, after reinvestigation of a maternal strain stored for two decades in the laboratory and do not have characteristic for reversion to virulent strain (KUTYREV,2009, SALTYKOVA and FAIBICH 1975, ISTC. 2006; DOMARADISKIJI. 1998, ANISIMOV. 1999, 2002). Currently the live attenuated vaccine which is commercially available in Russain and some of the states of FSU is known with its trade name *Vaccinum pestosum vivum siccum*, registration 93/160/21 and 93/132/20, for different routes of immunization included in regular vaccination calendar programs for the population living and working in enzootic regions (NIIEG. 2008, FEODOROVA et al. 2007,2008).

6) Differences in taxonomy- Based on the current knowledge of sequences of plague microbe isolated from different regions of the world indicates that, the widely used classifications of plague

microbe in the Western hemisphere that was based on minor phenotypic and biochemical differences in three biovars is “aniquated”. It does not fulfil the current global know-how on plague bacilli. The Russian APS established system of classifying on plague microbe based on numerical analysis of 69 phenotypic features in national level is used since 1987 plague research in the region that by far different from that of west is not included in International Code of Nomenclature of Bacteria, as a consequence, research publications in Russian language were /are rarely known outside the region. Although no system of plague microbe classification might be 100 % perfect, standardization of the nomenclature of plague to global level will be required. Currently the numerical classifications of plague microbes taxonomy used in FSU/CIS is more inclusive as compared to what is used currently in west.

Currently, there is no available animal or human vaccine trail against *B. mallei* and *B. pseudomallei* infection worldwide. Most of the available research publications on both pathogens are concentrated on pathogenesis, virulence factors, in elucidating the required immune systems for efficient protection, resistance to antibiotic therapy and route of transmission and distribution.
However various forms of approaches were made in attempt to develop vaccines, that includes killed whole cell vaccines, live attenuated vaccines, heterologous vaccines, acellular vaccines, subunit /recombinant DNA vaccines in various species of animal models (DANNENBERG and SCOTT 1958, LEVINE and MAURER 1958, VASILEIV et al. 1993, MANEZENYUK et al. 1999, ILYUKHIN et al. 2002,AMEMIYA et al 2002, WHITLOCK et al. 2008).

Based on cited publications, research strategies and attempts made to develop vaccines against *Burkoholderia mallei* and *B. pseudomallei* can be grouped in the following categories types:
1) Killed whole cell preparations : Nonviable capsulated or non-encapsulated killed cells offered no protection from a parenteral live challenged virulent strain of *B. malle*i (AMEMIYA et al. 2002). Strong responses of inflammatory mediators like IFN-γ, IL-18, IL-12, IL-27 and IL-6 expressions were observed in BALB/c mice with in 24 hrs after intraperitonial infection with B. mallei ATCC 2344 (ROWLAND et al. 2006). Immunization of BALB/c with IL-12 combined with non-viable *B. mallei* cells showed partial protection when compared vaccines based on killed cell. Indicating IL-12 shows an immunosimulatory effect, much of the enhancement is thought to occur via increased production of IFN- γ and activation of macrophages. In contrast SARKAR-TYSO (2009) recently reported that heat-inactivated *Burkholderia thailandensis*, *B. mallei* or *B. pseudomallei* cells as vaccines against murine melioidosis and glanders provided efficient protection against intraperitonial as in aerosol challenged with homologous strains and some cross protection were also observed when the mice were challenged with hetrologous strains. The Author explains the protection of heat killed vaccine in this study, might related to the much higher number of killed bacteria cells (10^8 cfu) used and differences in routes of administration.
2) Live attenuated virulent, mutant, low virulent or avirulent strains :-, Vaccines based seven selected auxotroph mutant strains of *B. pseudomallei* (Pur-, T^s) like *B. pseudomallie* C 141-206, provided over 70 % of protection for Balb/C mice, 30 % of protection for guinea pigs, 33 % of protection for white rats, and 25 % of protection for golden hamster against s.c. challenged doses of 1DLM (100 LD_{50} for Golden hamsters and 1000 LD_{50} for the rest of experimental animals) of

highly virulent *B. pseudomallei* strains. and the mean survival rates of immunized animals increased from 4 to 7 days in comparison to the control groups (ILYUKHIN et al. 1999). But no protection was provided against aerosol challenges That in fact indicates, besides to the species of animals, the level of protection of the vaccines depends on the the route of infection applied.

An attenuated branched-chain amino acid auxotroph mutants of *B. mallei* was also known shown in providing to partially protect against aersol infection (ULRICH. et al. 2005). Immunization with live attenuated *B. pseudomallei* like organism, *B. thailandensis*, not virulent for guinea pigs and slightly virulent for golden hamster, provided more than 50 % of protection for guinea pigs s.c. challenged with 200 LD_{50} against virulent strain of *B. pseudomallei* 100 (ILYUKHIN et al. 2002). Suggests, that live attenuated vaccine based on *B. thailandensis* as a potential prototype vaccine against melioidosis.

3) Non cellualr (surface) antigens and antigen complex:- Various surface antigens and antigen complexes extracted in different biochemical and Immunological methods studied examined in animal models showed. Among a number of fragments of antigens known in Burkholderia species, Immunization with 4 combined antigen complexes designated (A, D17, E13, and B4) showed some more protection (KALACHEV. 1997). Further studies are necessary, if vaccine based on surface antigens will be considered as another alternative of vaccine development against glnaders and melioidoisis, 4) DNA Vaccine:- Intramascularly adminstration of plasmid DNA enocoded flagellin protein against infection with *B. pseudomallei* in Balb-C/mice, was shown to elicit both, humoral and cellular immune responses, but in case of i.v challenges, a number of bacterial cells were detected in organs of the animals (CHEN et al. 2005). At the same time anti-flagella (flagellin) antibodies have been reported to be able to reduce bacterial motility *in vitro*, and thus provide some level of passive protection for diabetic rats infected with *B. pesudomallei* and can induce inflammatory mediators (BRETT et al. 1994). Suggests that vaccine based on flagellin protein alone, cannot protect melioidosis, but inclusion of flagellin protein in the development vaccine against melioidosis can enhance the efficacy of a vaccine in amplify the overall immune performance of the host after infection, 5) Subunit vaccines:-. From specific selected protein, categorized as subunit vaccine, the *LolC* - membrane protein, in adjuvant form, were found to be the most promising vaccine candidate against melioidosis. It was observed that five out of six Balb/ C mice immunized with *LolC* were protected against intra peritoneal infections with heterologous strains of *B. pseudomallei* (HARLAND et al. 2007). If the protein can also protect against pneumonic melioidosis or cross-protects against *B. mallei* infections is not known. .

Bip proteins - are combinants of type III secretion system, critical for the virulence of the pathogen, but the author did not found that they are protective antigens because vaccinated mice with any of the Bip proteins (like BipB, Bip C or Bip D) did not result in protecting against experimental melioidosis (DRUAR et al. 2008).

Although type IV pilin is known as highly immunogenic antigens and successfully used as subunit in prevention of several disease, vaccine trail in murine model failed to protect from lethal aerosol challenges of *B. mallei*. (FERNANDES et al. 2007), Currently, vaccinology will greatly benefit from

the emerging genomics technology such as bioinformatics, proteomics and DNA microarray. Information about the whole genome sequence of a pathogen, its' molecular structure and its' specific function will help us to better understand the organism and to identify possible targets for diagnosis, treatment and furthermore vaccine production. Thus new vaccine development against glanders and melioidosis can profit from it.

Concluding remarks

It was first in the year 1997 in Proceedings of the Scientific and Practical Conferences held in Saratov, Russia, dedicated to the centenary of the Russian Anti-Plague service, that experts from Western hemisphere get an opportunity, to participate in scientific conferences of this form after over six decades isolation of East and West blocs during cold war era.

This meeting was remembered by some experts in the West, with high remarks like, "On the biological side we are very far behind, there is a whole of history of things we on this planet we don't even know about" or in journal titled as " to fight plague, look the Russian's past" and in generalized form. The microbial science and research activities of the FSU /CIS on agents of bioweapon (military) and bioterrorism interest like plague and burkholderia species are continuous for decades as it is known " the enclosed world of secret microbial science." The agents belongs to the forefront research object of FSU, with high security, secrecy and intensity no state or institution is comparatively engaged with these agents in this level Worldwide.

From Russian point of view some of the pioneer researchers expresses as "The Soviets are born to research on Plague." which was based on the capacity of the APS which recruited hundreds of highly qualified experts, worked in various research institutes located in the region. The APS has more than a century old, experiences, monitoring, preventing disease outbreaks and research activities. What is today known about this pathogens are found in Russian language, publications and in Russian scientific journals which are rarely known in western world.

Yet, although changes have been seen in political-ideological arena, little moves are made to integrate these unique achievements of the API to the the global level. Misunderstandings misinterpretations, incorrectness of original research publications of Russian language are seen often in case cited in English language publications. The very majority of long year research published in Russian scientific journals are not known or unpublished literatures available in Russia still exists. For instance, in April, 2005 the U.S. Department of Defense announced a joint, multi-nation project arrangement between the U.S. Department of Defense (DoD), the Department of National Defence of Canada, and the Secretary of States for Defense of the United Kingdom of Great Britain and Northern Ireland was announced for the cooperative development of a vaccine to protect against plague. Under this agreement, the three nations will work together to develop and produce a plague vaccine that ultimately be licenced for human use. What every the reason might be Russia is not included. Exclusion of the immense knowledge available on the plague pathogen in

Russia in this particular case, may lead to a bottleneck to the global acceptance of the forthcoming plague vaccine based on the two immunogenic as well as virulence antigens F1 and V feasibility.

As the current concern of bioterrorism with the use of agents like Plague and Bukholderia grows and it is of a global concern, full understanding of research achievements of the FSU/CIS on the subject is indispensible. Today more than ever, global threats requires much greater collaboration between governments, experts, institutions, in creating a global grass–roots of networks of research and discussion, to achieve global solutions in developing, an efficient, universal, acceptable vaccine most importantly against pneumonic plague-the most possible way bioterrorism may take, and pathogenic Burkhoderia species that can have not only public health concerns but also security at the global level. Thus, a long term sustainable schedules of congresses and partnership between Russia, and other leading states on the research activities of the pathogens in concerns in the world will be the best option for the solution, development of prophylactic measures against the pathogens, in creating a think-tank, comprising ranges of experts from different countries of interest, initiating dialogs', enhancing transparency and better cooperation in both sides.

Integration of a tremendous amount of knowledge available in Russia is very essential in order to find a global solution to counter the modern and real treat of bioterrorism. A number of variable virulent strains, which are available, only in Russian laboratories and not but known outside FSU, needs to be accessed for researcher purpose to the international researchers. Based on the extent of the natural foci in the region and the genotypic and phenotypic diversity of strains available in the ecosystem, one can say that alone the natural foci in the region in itself is a natural laboratory for plague research and researchers worldwide. More over understanding more than a century old research activities of API, on the pathogens, its high competence, unique practical experiences is critical important in an attempt of developing vaccines against plague, glanders and melioidosis diseases.

6 SUMMARY

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VACCINE DEVELOPMENT AGAINST PLAGUE, GLANDERS AND MELIOIDOSIS IN THE FORMER SOVIET UNION IN COMPARISON TO THE CURRENT STATE OF GLOBAL KNOWLEDGE

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Keywords: plague, glanders, melioidosis, vaccine, biological weapon, antigens, immunity, *Yersinia pestis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, Anti-Plague Institutes, Former Soviet Union

The causative agents of plague (*Y. pestis*), glanders (*B. mallei*) and melioidosis *(B. pseudomallei*) are included in critical agents of bioterrorisim. They belong to the most intensively studied agents during cold war, specially in the former Soviet Union (FSU). Mostly what is known about these agents, particularly (*Y. pestis*) is not available in English language publications. Many of the studies are written in Russian language and published in Russian scientific journals. Thus, the work is designed to evaluate, published and unpublished Russian language written data obtained, in comparisions to the current state of global knowledge on the pathogens in concern.

The frame work of the dissertation is divided in to eight chapters: i) Introduction that includes historical backgrounds of the developments of biological weapon, vaccine development and peculiarities of the microbial science of FSU, ii) Challenging characteristics of the agents: including global distribution, taxonomical differences, exceptionality of the distribution of natural foci in the FSU and intraspecific diversity, multidrug resistance, potential as biological weapon and the importance of the development of vaccines, iii) Materials and Methods: the items used for this study were based on through survey of data search on the topic, retrieved sources of information that can be grouped to the following categories: a) International congresses and conferences: delegate handbooks and abstracts, public- workshops, b) Web-based searching engines, c) Personal communications to the authors and institutions per-email or per-post, d) Traditional mode of subscription of articles, books, bulletins, journals, e.t.c. based on library loans. Critical evaluations of the material were made based on updated knowledge of a vaccine required to combat agents categorized as critical biological weapon groups like plague, glanders and melioidosis. That includes safety, efficacy, quality, cost, production, stability, easy handling of the vaccine and its efficiency in human immunization based WHO or UNICEF recommendation standards. iv) Development of vaccines – describing the historical backgrounds of the development of plague vaccine licenced for human use with special emphasis on Live dried plague vaccine of EV

76 line NEIIG, the most common derivativ strain used in USSR and continued to be used in the countries of Former Soviet Union. Currently, It is the only available vaccine for human use, licenced in Russia against plague prophylaxis, compared to the new vaccine candidate based on two major proteins, the F1 and the LcrV antigen (recombinant or subunit) studies known in experimental animals and human trails, which is curently found in process of licensing, Various forms of plague vaccine studies are known in experimental animals such as heterologous vaccines, DNA based vaccines, e.t.c., which are also throughly analysed in this part. This section also includes known virulence factors versus immunogenicity of plague microbe and critically evaluate the role of major protein extracts currently known in experimental animal studies.

The second part of the vaccine section explores Several experimental studies made to develop vaccines against glander and melioidosis, for which currently ther is no vaccine avalaible. There are various forms vaccine trails known in experimental animal models that includes killed whole cell vaccines, live attenuated vaccines, heterologous vaccines, recombinant vaccines, acellular vaccines, DNA and subunit vaccines which are throughly evaluted in this part Although *B. mallei* and *B. pseudmallei* are genetically very similar, there are remarkable genetic variations with in the type strains, posses high genomic plasticity that complicates vaccine development that can provide sterile immunity. However, there are some evidence that shows killed vaccines do provide some protection against glanders and melioidosis depending on the routes of infections, types of strains, concentrations of the bacterial cells used e.t.c., But there is no evidence that shows a full protection.

Immunization based on avirulent strains of *B. thailandensis* demonstrated more than 50 % of protection in guinea pigs challenged s.c. with 200 LD_{50} of *B. pseudomallei* 100 virulent strain and which was proposed as a prototype against melioidosis. If similar protection can also provided against B. mallei is not yet known and further enhancements of its efficiency is required to achieve a sufficient protection.

Vaccines based on live attenuated *B. mallei* also showed some protection in experimental animals, but this approach alone will not likely seems to provide sufficient protection, but adding IL-2 as adjuvant improved the level of protection. Live vaccine based on less virulent strains might be the preferable approach, because the can induce both humoral and cellular immune response, which are critical for complete protection against *B. mallei and B. pseudomallei*. This section also critically evaluates the role of individual antigen or recombinant extracts currently know and thier level of protection in different sepcies of experimental animals, v) Discussion, vi) Summary, viii) References.

7 ZUSAMMENFASSUNG

Taye Kissi Jimma

IMPFSTOFFENTWICKLUNG GEGEN PEST, ROTZ UND MELIOIDOSE IN DEN STAATEN DER EHEMALIGEN SOWJETUNION IM VERGLEICH MIT DEM AKTUELLEN INTERNATIONALEN WISSENSSTAND

Institut für Tierhygiene und Öffentliches Veterinärwesen der Veterinärmedizinischen Fakultät der Universität Leipzig

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Die Erreger, welche die Pest (*Y. pestis*), Rotz (*B. mallei*) und Melioidose *(B. pseudomallei*) verursachen, gehören zu den bekanntesten bakteriologischen Waffen. Sie gehören besonders in den Staaten der ehemaligen Sowjetunion zu den am meisten und am intensivsten untersuchten Agenzien während des Kalten Krieges. Demzufolge sind die meisten Informationen bezüglich dessen was über diese Agenzeien bekannt ist, in Russischer Sprache verfasst und in englischsprachigen Publikationen wenig bekannt. Dementsprechend wurden in dieser Arbeit die wissenschaftlichen Publikationen in russischer Sprache im Vergleich zu dem aktuellen globalen Wissensstand analysiert.

Der Aufbau der Dissertation gliedert sich in acht Abschnitte: i) Einleitung, welche die historischen Hintergründe der Entwicklung von Biowaffen und Impfstoffen beinhaltet und Besonderheiten der sowjetischen mikrobiologischen Wissenschaften, ii) die Eigenschaften der Erreger, welche aktuell eine Herausforderung darstellen: die globale Verbreitung der Erreger, taxonomische Unterschiede, die spezielle Verbreitung der Naturherde in der ehemaligen Sowjetunion und deren intraspezifische Diversität, iii) Material und Methoden: das in dieser Arbeit verwendete Quellenmaterial wurde durch gründliche thematische Literaturrecherche gewonnen und kann den folgenden Kategorien zugeordnet werden: a) internationale Kongresse und Konferenzen: Handbücher, Abstracts, Veröffentlichungen von Workshops b) Web-basierte Suchmaschinen c) persönliche / direkte Kommunikation mit Autoren und Institutionen per Email oder Post d) traditionelle Bibliotheksausleihe (Artikel, Bücher, Bulletins, Journale, etc.).

Die kritische Auswertung des Materials erfolgte auf Basis des aktuellen Wissensstandes im Bereich der Impfstoffe, die gegen Agenzien zum Einsatz kommen, welche als kritische biologische Waffen eingestuft werden, wie z.B. die Pest, Rotz und Melioidose. Die Anforderungen an die Impfstoffe sind Sicherheit, Effizienz, Qualität, Kosten, Produktion, Stabilität, leichte Handhabung und Effizienz bei

der Immunisierung von Menschen, welche auf Standards basieren, die von der WHO oder der UNICEF empfohlen werden. iv) Impfstoffentwicklung – Beschreibung der historischen Hintergründe der Entwicklung eines Impfstoffes gegen die Pest, der für den Einsatz am Menschen zugelassen ist. Besonders berücksichtigt wurde der lebend abgetrocknete Pest-Impfstoff EV76 NIIEG Linie, der nur in Russland lizensiert ist im Vergleich zum Subunit /Rekombinanten Impfstoff in Versuchstieren. Der lebend abgetrocknete Pest Impfstoff, auf Basis von *Y. pestis* Linie NEIIG ist aktuell der einzig verfügbare Impfstoff, um die Pest in der Region zu bekämpfen. Er wird in den Ländern der ehemaligen Sowjetunion benutzt und ist bekannt für die Verminderung der Morbidität und Mortalität in Menschen bei der Pest. Zur Zeit gibt es in Russland Studien auf experimenteller Ebene mit einem verbesserten Impfstoff auf Basis des EV76 NEIIG Stammes mit geringer Toxizität und Reaktogenität. Im Westen befindet sich der rekombinante/subunit Impfstoff auf Basis von F1 und V Antigen im Prozess der Lizenzierung, aber es ist nicht bekannt, ob der Impfstoff in der Lage ist, einen universellen Schutz zu bieten und die Mortalität und Morbidität beim Menschen zu reduzieren.

Im zweiten Teil des Abschnittes werden die Versuche der Entwicklung eines Impfstoffes gegen Rotz und Melioidose untersucht. Es gab verschiedene experimentelle Studien mit Tierversuchs-Modellen zur Entwicklung von Impfstoffen gegen Rotz-und Melioidose-Erkrankungen, in welchen Impfstoffe mit ganzen getöteten Zellen, lebend attenuierten, heterologen, rekombinanten, azellulären, DNA und Subunit getestet wurden. Es gibt zwar einige Hinweise, die zeigen, dass diese Impfstoffe einen gewissen Schutz gegen Rotz und Melioidose bieten, jedoch ist dieser vom Infektionsweg abhängig, von den Typen der Stämme, der Konzentration der bakteriellen Zellen u.s.w.. Es gibt keine Nachweise, die auf einen vollständigen Schutz hindeuten. Obwohl *B. mallei* und *B. pseudomallei* genetisch sehr ähnlich sind, gibt es bemerkenswerte genetische Variationen innerhalb der Typen der Stämme, eine breite genomische Plastizität. Es sind intrazelluläre Bakterien, welche Impfstoffentwicklung erschweren. Impfstoffe müssen in der Lage sein, beide Zweige des Immunysytems zu induzieren, welche wichtig für einen kompletten und universellen Schutz sind.

Eine Immunisierung, die auf avirulenten *B. thailandensis*-Stämmen basiert, zeigte mehr als 50% Schutz bei Meerschweinen gegen subkutane Infektion mit 200LD_{50} virulente *B. pseudomallei* 100. Dies wurde als Prototyp für einen Impfstoff gegen Melioidose vorgeschlagen. Es ist noch nicht bekannt, ob der Impfstoff einen vergleichbaren Schutz gegen *B. mallei* bietet und es sind weitere Steigerungen seiner Effizienz nötig, um einen belastbaren Schutz zu erreichen.

Impfstoffe, die auf attenuierten *B. mallei* basieren, zeigen partiellen Schutz bei Versuchstieren, aber diese Vorgehensweise allein wird wahrscheinlich nicht genügen, um ausreichenden Schutz zu gewährleisten. Jedoch erhöhte die Zugabe von IL-2 als Adjuvans die Wirksamkeit. Lebend-Impfstoffe, die auf weniger virulenten Stämmen basieren, mögen der zu bevorzugende Ansatz sein, weil sie sowohl eine humorale als auch eine zelluläre Immunantwort auslösen welche wichtig sind für einen kompletten Schutz gegen *B. mallei* und *B. pseudomallei*. Aktuell ist gegen Rotz und Melioidose kein Impfstoff verfügbar. v) Diskussion, vii) Zusammenfassung, viii) Literatur-verzeichnis.

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